# Using comparative genomics to identify virulence traits and vaccine candidates in *Mannheimia haemolytica*

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfilment of the Requirements
For the Degree of Doctor of Philosophy
In the Department of Large Animal Clinical Sciences
Western College of Veterinary Medicines
University of Saskatchewan
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**Abstract**

Bovine respiratory disease (BRD) is the principal cause of morbidity and mortality among feedlot cattle. *Mannheimia haemolytica* is consistently implicated in this condition, but treatment options are diminishing with the rise of antimicrobial resistance and intensifying consumer pressure to reduce reliance on conventional therapies. Thus, sustainable alternatives like vaccination are required. In this study, the phenotypic and genotypic diversity of BRD pathogens were examined with the objective to identify vaccine targets using reverse vaccinology, an innovative approach to identify antigens via genomic sequence. Preliminary surveillance confirmed *M. haemolytica* serotype 2 isolates were predominant in healthy animals (75.5%) while serotypes 1 (70.7%) and 6 (19.5%) were common in diseased animals. Pathogens of BRD, including *M. haemolytica*, *Pasteurella multocida* and *Histophilus somni* were also isolated from North American BRD mortalities, and compared using pulsed-field gel electrophoresis and antimicrobial susceptibility. Concurrently, polymerase chain reaction detection of bacterial and viral agents confirmed that *M. haemolytica* with bovine viral diarrhea virus were the most prevalent. Whereas isolates from live cattle were found to have a relatively low level of resistance, several pathogens from the mortalities were found to contain integrative conjugative elements (ICE) conferring resistance to seven antimicrobial classes. These ICEs were transferred via conjugation to other bacterial species, emphasizing the need for alternative antimicrobial therapies. Collectively, data from these investigations informed the selection of 11 diverse *M. haemolytica* strains for whole genome sequencing and comparative analyses. Several bacteriophage associated genes and CRISPR-Cas regulated gene expression systems were identified and are likely contributing to virulence in *M. haemolytica*. Coding sequences across all genomes were screened using pan-genome analysis, identifying 291 candidates with cell-surface associated signatures. Using a cell-free translation system and enzyme-linked immunosorbent assay the candidates were screened against serum from cattle challenged with serovar 1, 2 or 6 of *M. haemolytica*, and ranked according to immunogenicity. The top five vaccine candidates included Ssa1, ComE, a solute binding protein, an outer membrane protein, and the periplasmic component of an ABC transporter. With further characterization, these unique antigenic candidates could be developed into a vaccine to effectively reduce the dependence on antimicrobial therapies.

**Acknowledgments**

I would like to gratefully acknowledge my graduate studies supervisors, Drs. Tim McAllister and Steve Hendrick for all of their guidance, support and patience. I would also like to acknowledge my advisory committee including Drs. Andrew Potter, Trevor Alexander and Gregory Penner. I appreciate all of the time and wisdom each member provided during the course of this project. Thank you to all of the technical support staff at Agriculture and Agri-Food Canada including Dawn Gray, Ruth Barbieri, Fred Van Herk, Wendi Smart and Lyn Patterson. To Ronda Carlson, Cindy Johnson and Bernie Genswein from the IT department at the Lethbridge Research Center, I cannot express enough appreciation for all of the support given. To Dr. Rahat Zaheer and Shaun Cook, who have both been so integral to the work done here, I express the most heartfelt gratitude. To all of the students and support staff that helped pick me up along the way, thank you so much for all of your time, effort and encouragement. Sample collection for this study was facilitated by Feedllot Health Management Services; their contributions are very much appreciated.

I gratefully acknowledge the financial support provided by Alberta Livestock and Meat Association and The Alberta Livestock Genome Program.

**Dedication**

To all of my family who have supported me in countless ways for many, many years. And, for Pam, Justin, Alicia and Paul C. You guys are the best anyone could ask for.

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**List of Abbreviations**

| | |
|---|---|
| AA | amino acid |
| AB | Alberta |
| ABC | ATP-binding cassette transporter |
| AMP | ampicillin |
| AMR | antimicrobial resistance |
| ATP | adenosine triphosphate |
| BHI | brain heart infusion |
| BLAST | basic local alignment search tool |
| BLP | bacterial lipoprotein |
| BHV-1 | bovine herpes virus 1 |
| BHV-4 | bovine herpes virus 4 |
| $BPI_3V$ | bovine parainfluenza 3 virus |
| BRD | bovine respiratory disease |
| BRSV | bovine respiratory syncytial |
| BVDV | bovine viral diarrhoea virus |
| BVDV1 | bovine viral diarrhoea virus 1 |
| BVDV2 | bovine viral diarrhoea virus 2 |
| Cas | CRSIPR associated proteins |
| CDS | coding sequences |
| cfu | colony forming units |
| CLSI | Clinical and Laboratory Standards Institute |
| CRISPR | clustered regularly interspaced short palindromic repeats |
| crRNA | CRISPR ribonucleic acid |
| CTC | chlortetracycline |
| cwt | hundred weight |
| DANO | danofloxacin |
| DNA | deoxyribonucleic acid |
| DR | direct repeat |
| dsDNA | double stranded deoxyribonucleic acid |
| DTH | delayed type hypersensitivity |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| ELISPOT | enzyme-linked immunospot assay |
| ENRO | enronfloxacin |
| EU | European Union |
| FFN | florfenicol |
| GEN | gentamicin |
| HCl | hydrogen cholride |
| HS | *Hisotphilus somni* |
| IBRV | infectious bovine rhinotracheitis virus |
| ICE | integrative conjugative element |

| | |
|---|---|
| IL | interleukin |
| IMD | invasive meningococcal disease |
| IMG | integrated microbial genomes platform |
| INFɣ | interferon ɣ |
| LB | Luria-Bertani |
| LDA | limiting dilution assays |
| Lkt | leukotoxin |
| LPS | lipopolysaccharide |
| MB | *Mycoplasma bovis* |
| MenB | *Neisseria meningitidis* serogroup B |
| MGE | mobile genetic elements |
| MH | *Mannheimia haemolytica* |
| MHC | major histocompatibility complex |
| MIC | minimum inhibitory concentration |
| MLV | modified-live vaccines |
| MST | minimum spanning tree |
| NaCl | sodium chloride |
| ncp | non-cytopathic |
| NE | Nebraska |
| NEO | neomycin |
| $Ni^{+2}$ | nickel |
| nsSNP | nonsynonymous single nucleotide polymorphism |
| OD | optical density |
| OXYT | oxytetracycline |
| PAM | proto-spacer-associated motif |
| PCR | polymerase chain reaction |
| PEN | penicillin |
| PFGE | pulsed-field gel electrophoresis |
| PGAAP | NCBI Prokaryotic genome annotation pipeline |
| PGAP | pan-genomes analysis pipeline |
| PI | persistently infected |
| PM | *Pasteurella multocida* |
| RDF | recombination directionality factor |
| $Rif^{R}$ | rifampicin resistant |
| RNA | ribonucleic acid |
| RNAseq | RNA sequencing |
| rRNA | ribosomal ribonucleic acid |
| RT-PCR | reverse transcription polymerase chain reaction |
| RV | reverse vaccinology |
| S1 | serotype 1 |
| S2 | serotype 2 |
| S6 | serotype 6 |

| | |
|---|---|
| SARS | severe acute respiratory syndrome |
| SNP | single nucleotide polymorphism |
| SPT | spectomycin |
| ssRNA | single stranded ribonucleic acid |
| T4SS | type 4 secretion system |
| TE | tris ethylenediaminetetraacetic acid |
| $T_{FH}$ | follicular helper T cells |
| TGF-β | transforming growth factor beta |
| $T_H$ | T helper |
| TIL | tilmicosin |
| TMP-SMX | trimethoprim/sulfamethoxazole |
| $T_{reg}$ | regulatory T cells |
| tRNA | transfer ribonucleic acid |
| TSA | tryptic soy agar |
| TUL | tulathromycin |
| TX | Texas |
| USA | United States of America |
| VSP | variable surface proteins |
| XNL | ceftiofur |

# 1 Literature review

## 1.1 Introduction to bovine respiratory disease and the North American cattle industry

### 1.1.1 Occurrence and economics

Bovine respiratory disease (BRD) is the most common and costly disease in North American feedlots. In spite of extensive study the condition is still responsible for up to 80% of morbidities and 50% of feedlot cattle mortalities (Sanderson et al., 2008), costing global beef production 3 billion USD$ annually (Watts and Sweeney, 2010), with 1 billion US$ of this loss occurring in the North America (Griffin, 2010; Taylor et al., 2010; Watts and Sweeney, 2010). Economic losses occur primarily through reduced carcass yield, the cost of preventative treatment or direct therapy and death (Griffin, 2010; Loneragan et al., 2001).

Bovine respiratory disease can refer to both acute and chronic infection of the respiratory tract of cattle. When observed in newly received calves within 60 days of arrival at the feedlot (Gagea et al., 2006), it is also called shipping fever. Although variable in severity, typical symptoms include depression, anorexia, fever, increased heart rate and rhinitis resulting in mucosal discharge or a dry encrusted muzzle, lacrimation and cough (Zecchinon et al., 2005). Early stages of infection show increased respiration followed by dyspnea (Zecchinon et al., 2005). Although BRD can occur throughout the year, shipping fever varies seasonally, typically peaking in recently weaned calves in the fall or early winter (Dabo et al., 2007; Loneragan et al., 2001; Ribble et al., 1995).

### 1.1.2 Bovine respiratory disease complex

Bovine respiratory disease is a complex condition, comprised of multiple factors including host status, environmental influences and microbial agents. Stressors associated with weaning, marketing and transport often lead to viral infections in predisposed or immune supressed animals. These infections further compromise natural clearance mechanisms in the respiratory tract and lead to bacterial infection in the lower lungs.

#### 1.1.2.1 Viral agents of BRD

##### 1.1.2.1.1 Bovine herpes virus 1 (BHV-1)

Multiple viral agents are associated with BRD, the most common being bovine herpes 1 (BHV-1), bovine viral diarrhoea virus (BVDV), bovine parainfluenza 3 virus ($BPI_3V$) and bovine respiratory syncytial virus (BRSV). Bovine herpes virus 1 is a double stranded DNA virus responsible for infectious bovine rhinotracheitis, characterized by acute inflammation with erosion and ulcers in the upper respiratory tract (Ellis, 2009; Yates, 1982). First documented in Canada in the late 50's, BHV-1 is now ubiquitous,

highly contagious and in addition to BRD can cause conjunctivitis, abortions, encephalitis and systemic infections.

Infections with BHV-1 typically occurs 2-7 days post exposure and uncomplicated cases do not manifest as pneumonia unless accompanied by a secondary bacterial infection (Yates, 1982). The presence of BHV-1 down regulates type 1 interferon and expression of both major histocompatibility complex (MHC) I and MHC II molecules, reducing the ability of the immune system to clear infected cells and display antigens (Ellis, 2009). BHV-1 can also enhance the pathogenesis of *Mannheimia haemolytica* during co-infection as the presence of the virus has been shown to increase the expression of the $\beta_2$ integrin CD11a/CD18 on bovine leukocytes (Ellis, 2009), which target leukotoxin secreted by the bacterium (Lawrence et al., 2008).

**1.1.2.1.2 Bovine respiratory syncytial virus (BRSV)**

Bovine respiratory syncytial virus is a single stranded RNA virus that can induce bronchointerstitial pneumonia, independent of a secondary bacterial infection (Taylor et al., 2010; Valarcher and Taylor, 2007). It can be responsible for 70% of respiratory disease in cattle with mortalities ranging from 2-20% (Gershwin, 2007). Infection doesn't advance beyond the respiratory epithelium (Ellis, 2009) and viral shedding begins 3 days after infection, with the vi0rus typically being undetectable after 10 days (Gershwin, 2007). The development of effective vaccines against BRSV is a challenge as the virus induces cytokine responses that result in the activation of the humoral or antibody mediated immune responses. This T helper 2 response targets extracellular parasites, bacteria, allergens and toxins rather than the desired T helper1 responses that activates the bactericidal activity of macrophage and is effective against intracellular pathogens, including viruses. As seen with BHV-1, the presence of BRSV contributes to the pathological effects of *M. haemolytica* by enhancing secretion of proinflammatory cytokines (Ellis, 2009) and enhancing leukocyte recruitment.

**1.1.2.1.3 Bovine parainfluenza 3 virus (BPI3V)**

Bovine parainfluenza 3 virus is a single stranded RNA virus of the *Paramyxoviridae* family that is endemic to cattle worldwide. Infection is common and generally subclinical (Ellis, 2009). Typically with BRD, $BPI_3V$ infections are coupled with a secondary bacterial agent. Acute infection results in loss of ciliated cells but also includes a marked reduction in the cytotoxic capabilities of infected macrophage (Ellis, 2010). Current epidemiological data is largely absent for $BPI_3V$ as it is endemic and difficult to detect through isolation (Henrickson, 2003). It is considered a catalyst to BRD and is already included in many BRD combination vaccines (Ellis, 2010).

#### 1.1.2.1.4 Bovine viral diarrhoea virus (BVDV)

Bovine viral diarrhoea virus is a single stranded, RNA pestivirus responsible for respiratory infection and reproductive disorders in cattle (Ridpath, 2010). Infection is spread largely by persistently infected animals, often as a result of infection of the fetus in early gestation (Lanyon et al., 2014). Bovine viral diarrhoea virus is divided into two genotypes, BVDV1 and BVDV2, based on antigenic and genetic differences and further into cytopathic and non-cytopathic (ncp) biotypes based on a strains ability to induce cell death in cell culture (Vilcek et al., 2005). Cytopathogenicity is not necessarily an indicator of virulence as many disease-causing strains are ncp, with only the ncp biotype is able to establish persistent infection in the fetus (Peterhans et al., 2010). Both acute and postnatal BVDV infections can contribute to BRD, mainly by facilitating secondary infection through immunosuppression and synergism with other pathogens (Ridpath, 2010). Acute infection results in damage to the epithelial surface in respiratory tract and depletion of lymphoid tissues, however, 70-90% of cases of BVDV infection are considered subclinical (Ridpath, 2010).

### 1.1.2.2 Bacterial agents of BRD

#### 1.1.2.2.1 *Mycoplasma bovis*

Multiple bacteria can contribute to BRD, the most common being *Mycoplasma bovis*, *Histophilus somni*, *Pasteurella multocida* and *Mannheimia haemolytica*. *Mycoplasma bovis* is a member of the class Mollicute and is associated with respiratory disease, mastitis, and arthritis in cattle (Caswell et al., 2010). First isolated in the USA in 1961, it is now detected worldwide (Horwood et al., 2014) with incidence ranging from complete absence to involvement in 90% of cases (Griffin, 2010). However, it is often found in the deep respiratory system and is typically only detected using serological methods (Griffin, 2010). Although acknowledged to contribute to acute respiratory disease it is often associated with chronic bronchopneumonia (Horwood et al., 2014), which is characterized by a caseonecrotic pneumonia that differs from the acute fibrinous pneumonia seen with classical BRD. Much about the etiology of *M. bovis* virulence is unclear, as the mechanisms by which it causes tissue damage are not well understood (Caswell and Archambault, 2007). Variable surface proteins (VSP) are the most characterized virulence factors, consisting of a family of lipoproteins with varied expression profiles that allow this bacterium to both adhere to different cell types and to evade host antibody responses (Caswell and Archambault, 2007; Caswell et al., 2010). Other virulence factors include production of a polysaccharide toxin, hydrogen peroxide, heat shock proteins and biofilms (Caswell and Archambault, 2007).

#### 1.1.2.2.2 *Histophilus somni*

*Histophilus somni* is a bacterium from the *Pasteurellaceae* family, responsible for multiple syndromes in cattle including thromboembolic meningoencephalitis, arthritis, pneumonia and reproductive disorders (Corbeil, 2007; Pérez et al., 2010). Pneumonic isolates are capable of inducing respiratory infection after intra-tracheal inoculation, but preputial and encephalitic strains fail to do so (Pérez et al., 2010). A commensal in the respiratory and genital tract, *H. somni* survives better in the bronchoalveolar areas than in nasal mucosa (Pérez et al., 2010) and is capable of surviving phagocytosis by macrophage, persisting within polymorphonuclear leukocytes for brief periods of time (Corbeil, 2007). *Histophilus somni* pneumonia is typically fibrinous suppurative and contributes to bronchopneumonia through a chronic progression as it does not disseminate rapidly through the lung as does *M. haemolytica* (Pérez et al., 2010). Multiple virulence factors have been identified in *H. somni* including lipooligosaccharide, immunoglobulin binding proteins, outer membrane and major outer membrane proteins and exopolysaccharides (Corbeil, 2007).

#### 1.1.2.2.3 *Pasteurella multocida*

*Pasteurella multocida* is also a member of the *Pasteurellaceae* family and is associated with several diseases in animals including cholera in birds, atrophic rhinitis in swine, septicaemia in rabbits and pneumonia in cattle (Blackall and Miflin, 2000; Wilson and Ho, 2013). Its role in bovine respiratory disease is more apparent with enzootic calf pneumonia in diary calves (Dabo et al., 2007), although the proportion of fatal BRD cases in feedlots associated with *P. multocida* is increasing (Welsh et al., 2004). Of the five serogroups and sixteen serovars described, serogroup A causes the majority of BRD infections with serotype A3 being isolated from approximately 35% of BRD cases in the USA (Wilson and Ho, 2013). *Pasteurella multocida* may have synergism with *Mycoplasma* spp. as they are commonly isolated together (Dabo et al., 2007). However, *P. multocida* has been shown to inhibit the growth of *M. haemolytica* (Wilson and Ho, 2013). *Pasteurella multocida* harbors multiple virulence factors including a lipopolysaccharide (LPS) that confers resistance to serum complement, a cytotoxin, iron acquisition proteins, and filamentous hemagglutinin (Wilson and Ho, 2013).

#### 1.1.2.2.4 *Mannheimia haemolytica*

*Mannheimia haemolytica,* also a member of the *Pasteurellaceae* family, is the bacterium most commonly isolated from newly received calves suffering from pleuropneumonia (Confer, 2009). As a result, it is often considered the primary bacterial agent of BRD (Rice et al., 2008). Of the 12 capsular serotypes identified, serotype 1 (S1) and serotype 6 (S6) are the most prevalent in BRD (Al-Ghamdi et al., 2000; Rice et al., 2007). Serotype 2 (S2) is found frequently as a commensal in the upper respiratory

tract of healthy animals (Klima et al., 2011), but is associated with respiratory disease and causes high mortality in wild and domestic sheep (Lawrence et al., 2010).

*Mannheimia haemolytica* has several virulence factors. These include multiple fimbriae, adhesions, outer membrane proteins (OmpA) and surface lipoproteins (Lpp1) that assist in adherence and colonization (Confer, 2009). Neuraminidase and O-sialoglycoprotein endopeptidase also support attachment, modifying the cell surface and reducing respiratory mucosal viscosity and facilitating access to host epithelial cells (Griffin, 2010). Other factors like transferrin-binding proteins assist in scavenging iron for bacterial growth. The capsular polysaccharide of *M. haemolytica* can also protect against phagocytosis (Rice et al., 2007).

Lipopolysaccharide is a major component of the Gram-negative cell wall, contributing to the structural integrity of bacteria, but it is also an important virulence factor in *M. haemolytica*. As an endotoxin, LPS can induce inflammatory cytokine responses and expression of the $\beta_2$ integrin leukotoxin receptor on bovine leukocytes (Gioia et al., 2006). In addition, it forms complexes with leukotoxin that may help enhance its cytotoxicity (Rice et al., 2007). *Mannheimia haemolytica* serotypes are virtually clonal for LPS, with most of the minor variation occurring in the lipid A and oligosaccharide region (Davies and Donachie, 1996).

Leukotoxin (Lkt) is the primary virulence factor of *M. haemolytica* (Dassanayake et al., 2009). It is a pore forming cytotoxin of the RTX family that has affinity for the CD18 β subunit of β2 integrins on bovine leukocytes (Griffin, 2010). Actively secreted by all serotypes during log phase growth (Rice et al., 2007), Lkt activity is responsible for the lung lesions observed during *M. haemolytica* infection (Fedorova and Highlander, 1997; Tatum et al., 1998). Sequence diversity occurs between serovars, with S1 and S6 Lkts similar and S2 Lkts more variable (Davies et al., 2001; Rice et al., 2007). Low concentrations of Lkt enhance the inflammatory response, inducing cells to release free radicals, cellular proteases and proinflammatory cytokines (Confer, 2009; Griffin, 2010; Rice et al., 2007). At high concentrations Lkt, impairs leukocyte function by inducing osmotic swelling, pore formation and necrosis (Confer, 2009; Griffin, 2010). *Mannheimia haemolytica* pneumonia is characterized by acute cranioventral fibrinous to fibrinopurulent pleuropneumonia (Confer, 2009), with lesions that are lobar and anteroventrally distributed (Zecchinon et al., 2005) with neutrophil and fibrin accumulation in the lungs (Rice et al., 2007).

### 1.2 Management of BRD

#### 1.2.1 Preconditioning

Current management strategies for BRD consist of preconditioning programs, vaccination and antimicrobial therapies. Preconditioning is used to prepare calves to enter the feedlot and can include castration, dehorning, weaning, administration of antimicrobials, vaccination, and adaptation to dry feed and water troughs prior to transport (Schumacher et al., 2012). Preconditioned calves often have better performance with lower morbidity and mortality than those that have been weaned and immediately shipped to a feedlot. Preconditioned calves can be worth over $7/cwt US$ more than unconditioned calves with an added $2.37/cwt US$ if the health program is third party certified (Schumacher et al., 2012).

#### 1.2.2 Vaccination

Vaccination is an important tool in the management and prevention of BRD. The majority of cattle entering feedlots receive viral vaccination. In the USA a reported 95.1% of calves are vaccinated against BVDV, 93.2% against BHV-1, 61.4% against BRSV and 55.1% against $BPI_3V$ (Theurer et al., 2015). Despite a current lack of data to support the effectiveness of these products in a commercial settings, there is a consensus among most veterinarians that vaccination at arrival against BHV-1, BVDV BRSV and $BPI_3V$ (Theurer et al., 2015).

There are three major types of vaccines: inactivated, modified-live and subunit vaccines containing purified components of the infectious agent (Bambini and Rappuoli, 2009). Viral vaccines for BRD are usually modified-live vaccines (MLV) or contain inactivated virus antigens. Modified-live vaccines typically induce cell-mediated and humoral responses by activating T lymphocytes, generating an adequate immune response with one vaccination, while inactivated vaccines stimulate the humoral response to encourage B cell activity and antibody generation and thus require a booster (Theurer et al., 2015). There are many viral vaccines available either targeting single, multiple or all viral agents associated with BRD (Bowland and Shewen, 2000).

There are multiple commercial vaccines available against *M. haemolytica, P. multocida, H. somni* and *M. bovis* (Bowland and Shewen, 2000). These typically contain bacterins, or killed whole bacterium, with *M. haemolytica* vaccines typically supplemented with Lkt. Meta-analysis of data published surrounding the effectiveness of these vaccines indicates that there may be some benefit for vaccination against *M. haemolytica* and *P. multocida,* but at this stage there is no solid evidence supporting vaccination against *H. somni* (Larson and Step, 2012). However, the outcomes of these studies vary greatly, some reducing BRD morbidity, some showing no significant difference from controls, and others

potentially increasing the risk of morbidity (Larson and Step, 2012). Although natural *M. bovis* infection has been shown to elicit a robust immune response and both commercial and autogenous vaccines have been produced, there is also no evidence of their efficacy under field conditions (Caswell et al., 2010). Vaccination against bacterial agents in feedlots is less frequent than against viruses with approximately 66% of feedlots in the USA vaccinating against *H. somni* and *M. haemolytica* and 21.8% vaccinating against *M. bovis* (Neibergs et al., 2014).

**1.2.3 Antimicrobial Use**

Antimicrobials are a primary tool used against bacterial pneumonia in feedlots, either as preventatives or direct therapies, being administered in-feed or through injection. Shown to reduce BRD morbidity by 50% and mortality by 30-50% (Miles and Rogers, 2014), 20-50% of calves receive injectable antibiotics upon arrival at the feedlot (Checkley et al., 2010; Hilton, 2014). With over 80% of the antimicrobials licenced for use in cattle being used to control BRD (Bowland and Shewen, 2000), it is clear these therapies must be efficacious if the current intensive feedlot beef production systems in North America are to remain viable.

Unfortunately, even with the use of antibiotics, best management practices and vaccination programs, the prevalence of BRD has not fallen (Neibergs et al., 2014), but appears to actually be increasing (Hilton, 2014; Miles and Rogers, 2014). A likely contributor to this is the recent emergence of pan-antibiotic resistant *M. haemolytica* (Eidam et al., 2015; Lubbers and Hanzlicek, 2013) and *P. multocida* in beef cattle (Michael et al., 2012). Some of these isolates have been shown to harbour self-transmissible conjugative elements with extended multidrug resistance gene arrays (Michael et al., 2012) conferring resistance to the entire suite of drug therapies currently used to treat BRD, with the exception to ceftiofur (Lubbers and Hanzlicek, 2013; Michael et al., 2012; Miles and Rogers, 2014). The development of antimicrobial resistance on this scale is not only of concern for maintaining current therapies, but also poses a potential hazard to human health if these elements transfer into zoonotic pathogens.

With the extent of multidrug resistance occurring in BRD pathogens, new solutions to prevent and treat bacterial agents will need to be found either through altering management practices, the development of new drugs and/or the design of more effective vaccines. The development of antibiotics has declined substantially over the last thirty years (Prescott, 2014) with only three new drugs approved by the USDA between 2005 and 2009 (May, 2014). The current global market for antibiotics is low and with no incentive for development many pharmaceutical companies have shut

down pipelines and bowed out of antimicrobial production entirely (May, 2014), despite there being few alternatives to existing antibiotics.

**1.3 Using the Genome for Vaccine design**

While drug development has stalled, vaccine design has been transformed by advancements in genomic technologies. Whole genome sequencing and bioinformatics have allowed for rapid identification of novel antigen candidates previously unidentifiable by convention methods (Figure 1.1). This includes antigens that are less abundant, not expressed *in vitro* or not very immunogenic during infection (Bertholet et al., 2014). Genome mining is a step up from the classical approach of isolation, infection and injection with the causative agent as it has the potential to identify all of the proteins produced by an organism, including their cellular location and predicted function (Rappuoli, 2001). By combining *in silico* analysis of genome sequences with recombinant DNA technologies and structural and function genomics, targeted vaccines can be rapidly developed. A good example of this approach was witnessed with the emergence of severe acute respiratory syndrome (SARS) in 2003, where sequencing and bioinformatic analysis of the responsible coronavirus identified a protective vaccine antigen candidate in less than a month (Bambini and Rappuoli, 2009).

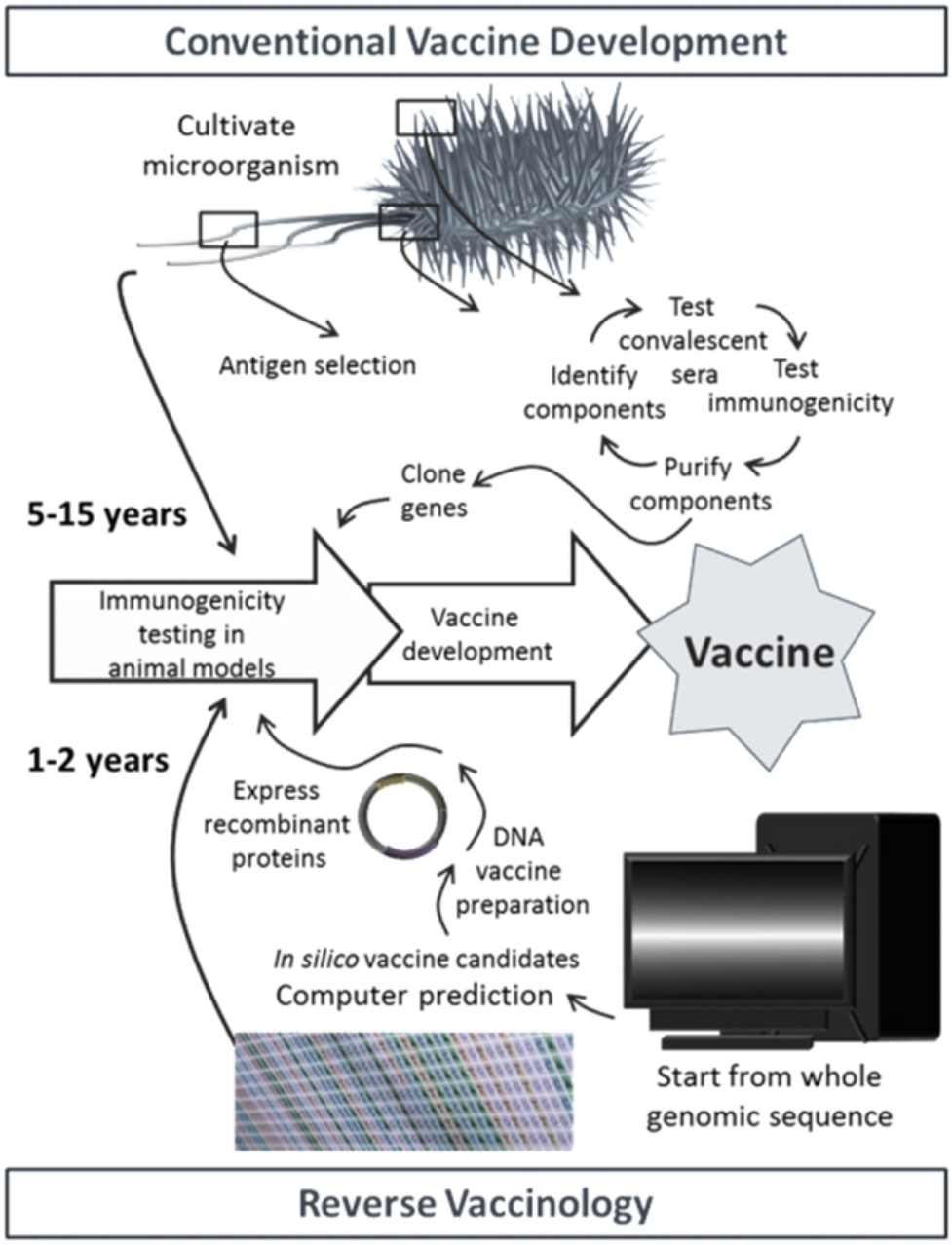


Figure 1.1 Reverse vaccinology vs conventional vaccine design. Reproduced with permission from Rappuoli, 2000 © Elsevier.

**1.3.1 Reverse vaccinology**

The strategy to design vaccines starting from the genome rather than the organism, termed reverse vaccinology (RV), was first applied to *Neisseria meningitidis* serogroup B (MenB) (Pizza et al., 2000). The causative agent of meningitis and severe sepsis, *N. meningitidis* is a challenge for conventional vaccine design as it displays strain variation and the capsular polysaccharide is both poorly

immunogenic and can induce autoimmunity (Rappuoli, 2001). To examine potential candidate proteins for vaccine use, the whole genome of a single *N. meningitidis* strain was sequenced and the coding genes examined for protein motifs that were exported to the cell surface, or embedded within the lipid bilayer (Pizza et al., 2000). A total of 600 candidate proteins were identified with 350 being expressed in *E. coli* and used to immunize mice (Pizza et al., 2000). The serum generated was used to identify surface expressed proteins that were conserved across strains and induced a bactericidal response (Pizza et al., 2000). The resulting five antigen candidates were combined to produce the multicomponent meningococcal serogroup B vaccine called 4CMenB (Bexsero®) which has now completed clinical trials (Donati and Rappuoli, 2013) and is approved for use in over 30 countries (Doolan et al., 2014) including the EU, USA and Canada (Medini et al., 2015).

Because the incidence of invasive meningococcal disease (IMD) is low, there is little available data to support the efficacy of the 4CMenB vaccine (Dwilow and Fanella, 2015). However, several clinical studies have examined its immunogenicity through surrogate markers, including serum bactericidal activity assays and a novel Meningococcal Antigen Typing System, all of which support the vaccine's effectiveness (Dwilow and Fanella, 2015; Gorringe and Pajón, 2012; Medini et al., 2015). Current measures used to determine the cost-effectiveness of its inclusion in national vaccine programs is hindering the adoption of 4CMenB (Dwilow and Fanella, 2015) with authorities in Canada recommending that only high risk individuals such as those that have contacted IMD-infected patients be vaccinated. This policy may change if the vaccine was demonstrated to have an effect on carriage or herd immunity, information that only could be garnered after its widespread use (Dwilow and Fanella, 2015).

Since its first application, reverse vaccinology has been used to identify vaccine protein antigens against several important pathogens including *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Bacillus anthracis*, *Porphyromonas gingivalis*, *Mycobacterium tuberculosis*, *Helicobacter pylori, Streptococcus agalactiae*, *E. coli*, *Leishmania major* and *Leishmania infantum* (Bambini and Rappuoli, 2009; Donati and Rappuoli, 2013). Not previously applied to agents contributing to BRD, the RV approach has the potential to identify novel antigens candidates that could be used to formulate protective vaccines against respiratory pathogens in cattle.

**1.4 Comparative genomics**

The advent of comparative genomics has improved upon the original RV approach. The genetic diversity of strains within a species can be substantial, due largely to horizontal gene transfer by mobile genetic elements (Bambini and Rappuoli, 2009). To develop a vaccine capable of providing broad

coverage, an understanding of the population structure of a pathogen is required (Donati and Rappuoli, 2013). Sequence comparison of pathogenic and non-pathogenic strains can assist in identifying those factors that contribute to virulence and enable the design of vaccines that target those strains that specifically cause disease.

### 1.4.1 Pan and core genome

Comparative genomics is employed to examine a range of features including overall sequence similarity, gene arrangement and gene transfer, but delineation of the pan and core genomes are key to using this approach in vaccine design. The pan-genome was first defined by Tettelin et al. (2005) as the global compliment of genes in a species that codes for the complete repertoire of proteins it can produce. Once identified, candidate proteins in the pan-genome can be analyzed using bioinformatic approaches for markers that export them to the cell surface, and are thus most likely to serve as targets for the host immune system. The core genome consists of the set of genes shared by all strains, and likely encodes for functions related to basic biology and phenotype and can provide a great deal of information about the evolution and lifestyle of a bacterial species (Donati and Rappuoli, 2013). Comparison of the core and pan-genome identifies vaccine candidates that can be used as antigens to provide protection against all strains within a species or against a subset of particularly virulent strains. Determining if the pan-genome is open or closed allows for evaluation of the minimal number of strains required to reasonably represent the species (Tettelin et al., 2008). An open pan-genome occurs where the addition of new strains to the dataset will contribute new genes to the database. A closed pan-genome occurs when the addition of new strains results in the number of strain specific genes converging at zero, at which point the dataset encompasses the number of genome sequences required to sufficiently characterize the species (Medini et al., 2005).

### 1.4.2 Mobile genetic elements

Approximately three quarters of all genes in bacterial genomes have been acquired through horizontal gene transfer of mobile genetic elements (MGE) (Juhas, 2015). These include plasmids, phage, genomic islands and genomic modules that transmit horizontally through conjugation, transduction or transformation (Wozniak and Waldor, 2010). Mobile genetic elements, including bacteriophage and integrative conjugative elements, can promote pathogenesis through the provision of virulence factors, so identification of these elements within genomes is important to understand the mechanisms of virulence and to identify targets for vaccine design in pathogenic strains.

#### 1.4.2.1 Bacteriophage

Phages are the most abundant organisms on the planet, and those that infect bacteria and integrate into the host chromosome can play key roles in diversification and the evolution of pathogens (Boyd and Brüssow, 2002). The horizontal transfer and acquisition of virulence factors through prophage is a major driving force in the emergence of pathogenic isolates not only through the provision of toxins, but also through factors that regulate virulence gene expression, adhesion, colonization and invasion (Wagner and Waldor, 2002). Phage-derived virulence factors are utilized by many important pathogens including *E. coli*, *Salmonella* spp., *Vibrio cholera*, *Pseudomonas aeruginosa*, *Listeria* spp., *Streptococcus* spp. and *Staphylococcus* spp. (Boyd and Brüssow, 2002), and are frequently responsible for the zoonotic phenotype of these bacteria (Penades et al., 2015).

#### 1.4.2.2 CRISPR-Cas systems

To protect against bacteriophage predation many bacterial species employ a CRISPR-Cas (clustered regularly interspaced short palindromic repeats/CRSIPR associated proteins) system. An adaptive and inheritable prokaryotic immune system, CRISPR-Cas is widespread, present in 40-70 % of bacteria (Deveau et al., 2010) and 90% of archaea (Sorek et al., 2008). Although not likely to be a direct target for vaccine development, CRISPR-Cas systems influence virulence by preventing bacteriophage from integrating into the genome and by regulating prophage and gene expression within the host.

CRISPRs are the most abundant family of noncoding sequences in prokaryotic genomes (Szczepankowska, 2012) and although first described in 1987 (Ishino et al.), it wasn't until 2005 that CRISPR-Cas systems were proposed to mediate immunity against extrachromosomal agents (Sorek et al., 2008) such as phages and plasmids (Makarova et al., 2011). Three major types and ten subtypes of CRISPR-Cas systems have been identified (Makarova et al., 2011), all consisting of a CRISPR arrays next to a series of 4 to 20 CRISPR associated (Cas) genes. The CRISPR array consists of a leader sequence (20-534 bp) followed by numerous conserved direct repeats (21-48 bp) that are interspersed with variable sequences called spacers (21-72 bp) (Sampson and Weiss, 2013; Szczepankowska, 2012). Spacers are typically derived from foreign genetic material within phage or plasmids and can number in the hundreds within an array (Barrangou and Horvath, 2012; Horvath and Barrangou, 2010; Szczepankowska, 2012). Approximately 50% of CRISPR containing genomes have more than one CRISPR locus with additional arrays often lacking Cas genes in their proximity (Swarts et al., 2012).

The CRISPR-Cas mechanism employs spacer acquisition in a process known as adaptation. In the type I and II systems, adaptation relies on CRISPR-Cas machinery to identify, bind and cleave invading nucleic acids that are incorporated as spacers into the CRISPR array (Makarova et al., 2011).

The selection of proto-spacers (spacer precursors) from the invading nucleic acid is dependent on the presence of a proto-spacer-associated motif (PAM), a short nucleic acid sequence located either downstream or upstream of the proto-spacer. Proto-spacer-associated motifs are not incorporated into the spacer itself, enabling the CRISPR-Cas system to distinguish between target proto-spacer and the host CRISPR array (Szczepankowska, 2012). Spacers are typically inserted sequentially, adjacent to the leader of the array, but occasionally spacer acquisition has been shown to occur internally within the array (Westra and Brouns, 2012).

The mechanism for CRISPR interference first requires transcription and processing of the array into a long precursor CRISPR RNA or pre-crRNA (Maraffini and Sontheimer 2010; Jore et al., 2011). The pre-crRNA is cleaved within the repeat sequences by a complex of Cas proteins called Cascade (CRISPR-associated complex for antiviral defense) to produce small crRNA that are further trimmed to generate mature crRNA (Maraffini and Sontheimer 2010; Jore et al., 2011). The complex of mature crRNA and Cascade, together with a Cas protein with nuclease activity, targets and cleaves invading viral or plasmid DNA with complementarity to the spacer sequence (Jore et al., 2011; Marraffini and Sontheimer, 2010).

The CRISPR-Cas system is widely recognized as a defense mechanism against foreign invasive plasmids and phage, but its role in gene expression and regulation of cellular processes including biofilm formation, lysogenization, spore formation, replicon maintenance and segregation, and DNA repair-recombination are also being examined (Szczepankowska, 2012). CRISPR spacers have been found to target chromosomal genes, and are thought to regulate transcription of host factors and in some cases influence virulence. A recent example has been described for *Francisella novicida,* a bacterium responsible for tularemia, where CRISPR down regulated an immunostimulatory bacterial lipoprotein (BLP) enabling the bacterium to avoid immune detection in the host (Sampson and Weiss, 2013). Although CRISPR itself is not a target for vaccine design, examination of those genes it targets can point towards candidates that may be central to virulence in pathogenic bacteria.

**1.4.2.3 Integrative Conjugative Elements**

Arguably, one of the most important families of mobile genetic elements in bacteria are integrative conjugative elements (ICEs). These elements are unique in that they are self-transmissible, encoding all of the mechanics required for integration, excision and transfer (Wozniak and Waldor, 2010). Acquisition of ICEs allows bacteria to rapidly adapt to changing environmental conditions and colonize new niches (Burrus and Waldor, 2004) by mediating the transfer of accessory genes that alter phenotype, including antimicrobial resistance and virulence (Wozniak and Waldor, 2010). As a result,

they are key contributors to bacterial pathogenicity and the shaping of bacterial genomes (Burrus and Waldor, 2004).

In the past ICEs were described as transposons, conjugative transposons, genomic islands and plasmids as they share characteristics in common with all of these elements (Seth-Smith et al., 2012). As the key components of ICEs are often found independently as components of other MGE, it can be difficult to identify them through bioinformatic approaches (Wozniak and Waldor, 2010). For example, T4SS can be found on the chromosome of bacteria not involved in conjugation but associated with protein transport. And, integrases are present in both transposons and prophage. However, with the continuing accumulation of whole genome sequencing data, it is now clear that ICEs are far more abundant than first anticipated (Seth-Smith and Croucher, 2009) and more prevalent than conjugative plasmids (Guglielmini et al., 2011).

All ICEs contain three modules ensuring their maintenance, dissemination and regulation (Burrus and Waldor, 2004). Maintenance modules contain an integrase that promotes integration, the majority belonging to the tyrosine recombinase family, although serine recombinases and DD[E/D]-transposases have also been described (Wozniak and Waldor, 2010). Excision requires the integrase gene, but also a recombination directionality factor (RDF) that is used to bias activity towards excision over integration (Wozniak and Waldor, 2010). Integrative conjugative elements can be lost if excision occurs during cell division or the element doesn't properly integrate back into the host after replication. As a result, some ICEs also encode mechanisms to ensure their stability, as is the case with toxin-antitoxin systems.

Toxin-antitoxin systems are small genetic modules that consist of a stable toxin and a liable, neutralizing antitoxin as its counterpart. When co-expressed, these form a stable complex which inhibits the toxin's activity. However, if this co-expression stops either through element loss or arrest of expression, the unstable antitoxin degrades while the toxin persists, resulting in cell death. Toxin-antitoxin systems are widespread in prokaryotes, and employed by MGE including plasmids, phage or genomic islands to ensure their maintenance. They also function within the bacterial genome to prevent the spread of phage through a mechanism called abortive infection (Yamaguchi et al., 2011). During phage infection host protein expression can be slowed or arrested. During this time, if toxin-antitoxin systems are active on the host chromosome, the antitoxin will degrade without replacement and the toxin will kill the infected cell. In this manner, the spread of the phage throughout the bacterial population is prevented.

Integrative conjugative elements disseminate through conjugation, using a model similar to that proposed for plasmids. A relaxase is required to nick DNA at the origin of transfer and to interact with

the coupling protein responsible for targeting the nucleoprotein complex to the mating pore for transfer (Wozniak and Waldor, 2010). A single stranded copy of the ICE is transferred into the recipient where it becomes a template for polymerase to rebuild the double stranded circular element (Wozniak and Waldor, 2010). However, some ICEs have been shown to undergo rolling circle replication and be transferred as double stranded DNA, while others are capable of autonomous replication (Leaves et al., 2000; Lee et al., 2010a; Lee et al., 2010b; Wozniak and Waldor, 2010).

To transfer ICE DNA, the majority of Gram-negative bacteria employ a conjugation apparatus similar to the type 4 secretion system (Wozniak and Waldor, 2010). In Proteobacteria this consists of an ATPase and mating pair formation genes, which code for a membrane spanning channel and an extracellular pilus that attaches to the recipient cell (Guglielmini et al., 2012). Based on recent phylogenetic analysis, T4SS in Proteobacteria can be characterized into one of four groups (Guglielmini et al., 2012) $MPF_T$ (modeled from the T-DNA conjugation system of *A. tumefaciens* plasmid Ti), $MPF_F$ (modeled from the plasmid F), $MPF_I$ (modeled from the IncI plasmid R64), and $MPF_G$ (modeled from ICEHIN1056) (Smillie et al., 2010). Less is understood about the conjugation systems used to transfer ICEs in Gram-positive bacteria although it has been shown that the T4SS is not needed to facilitate ICE transfer in *Streptomyces ambofaciens* (Wozniak and Waldor, 2010). The transfer system of ICEs can be hijacked by other MGE (Burrus and Waldor, 2004) and as a result ICEs can play a secondary role in facilitating the spread of virulence factors carried on transposons, genomic islands and plasmids.

Many ICEs are not host specific and can be transferred to a wide range of species (Garriss et al., 2009), but the factors determining host range have not been explored in detail (Wozniak and Waldor, 2010). Like other mobile genetic elements, ICEs have been identified that use entry exclusion systems to prevent host cells from obtaining other closely related elements (Wozniak et al., 2009). Integrative conjugative element regulation mechanisms are also largely undefined but those that have been characterized are diverse (Burrus and Waldor, 2004). Some ICEs are regulated through a repressor similar to that found in lambda phage. For these ICEs, DNA-damaging agents that induce an SOS-like response, such as exposure to mitomycin C and quinolones will increase their transfer rates (Wozniak and Waldor, 2010). Other ICEs appear to be stimulated by sub-inhibitory concentrations of tetracycline (Bellanger et al., 2014; Burrus and Waldor, 2004) or through quorum sensing (Wozniak and Waldor, 2010).

In addition to the functional modules necessary for ICE mobility and persistence, many of these elements carry accessory cassettes that encode virulence factors including genes that can biosynthesize antimicrobials, degrade toxic aromatics and confer resistance to antimicrobials, heavy metals or phage

(Burrus and Waldor, 2004). Integrative conjugative elements have also been found that enhance colonization, fix nitrogen and promote biofilm formation (Wozniak and Waldor, 2010). Accumulation of accessory cassettes in ICEs can be mediated by transposons or by homologous recombination (Wozniak and Waldor, 2010).

Integrative conjugative elements have been identified in *M. haemolytica, P. multocida* (Eidam et al., 2015; Michael et al., 2012) and *H. somni* (as described in Chapter 2) and in addition to having important implications for antimicrobial resistance (AMR), genes coding for the extracellular components forming the pilus of the T4SS are possible targets for vaccine design as they are linked to enhanced virulence and are readily accessible by the host immune system. As more ICEs are identified, classification schemes are being designed with sequence similarities in integrase genes, integration site and organization of T4SS arrays, proposed as a means of describing element relationship (Guglielmini et al., 2011). A common identification scheme will likely assist in characterizing the movement of genes coding for antibiotic resistance among bacterial communities.

**1.5 Vaccines and Evaluation of Vaccine Candidates**

Vaccination is one of the most efficient and cost-effective approaches to controlling infectious diseases (Doolan et al., 2014; Rappuoli and Aderem, 2011). Although RV and advancements in "omics" technologies have revolutionized vaccine design, evaluations of the specific immune responses induced by antigens is still required to determine the efficacy of vaccines. A step in this process is determining which arm of the adaptive immune responds to the selected antigen.

**1.5.1 The two arms of the immune response**

The immune system can be divided into two main subsystems, the innate and the adaptive. Detailed reviews of each system can be found elsewhere (Kennedy and Poland, 2011; Luckheeram et al., 2012; McVey and Shi, 2010), so the main cellular components are only briefly described here and presented in Figure 1.2. The innate immune system is the first line of defence against pathogenic agents and encompasses multiple protective measures including anatomical and physiological barriers, the compliment pathway, and the activities of mononuclear phagocytes and granulocytic cells that induce inflammatory responses. Innate responses are not specific to a particular agent, but are induced by conserved molecular patterns found on all microorganisms and as a result it can act rapidly. The innate system may eradicate pathogens independently or it may stimulate the adaptive system to become active in controlling the infection.

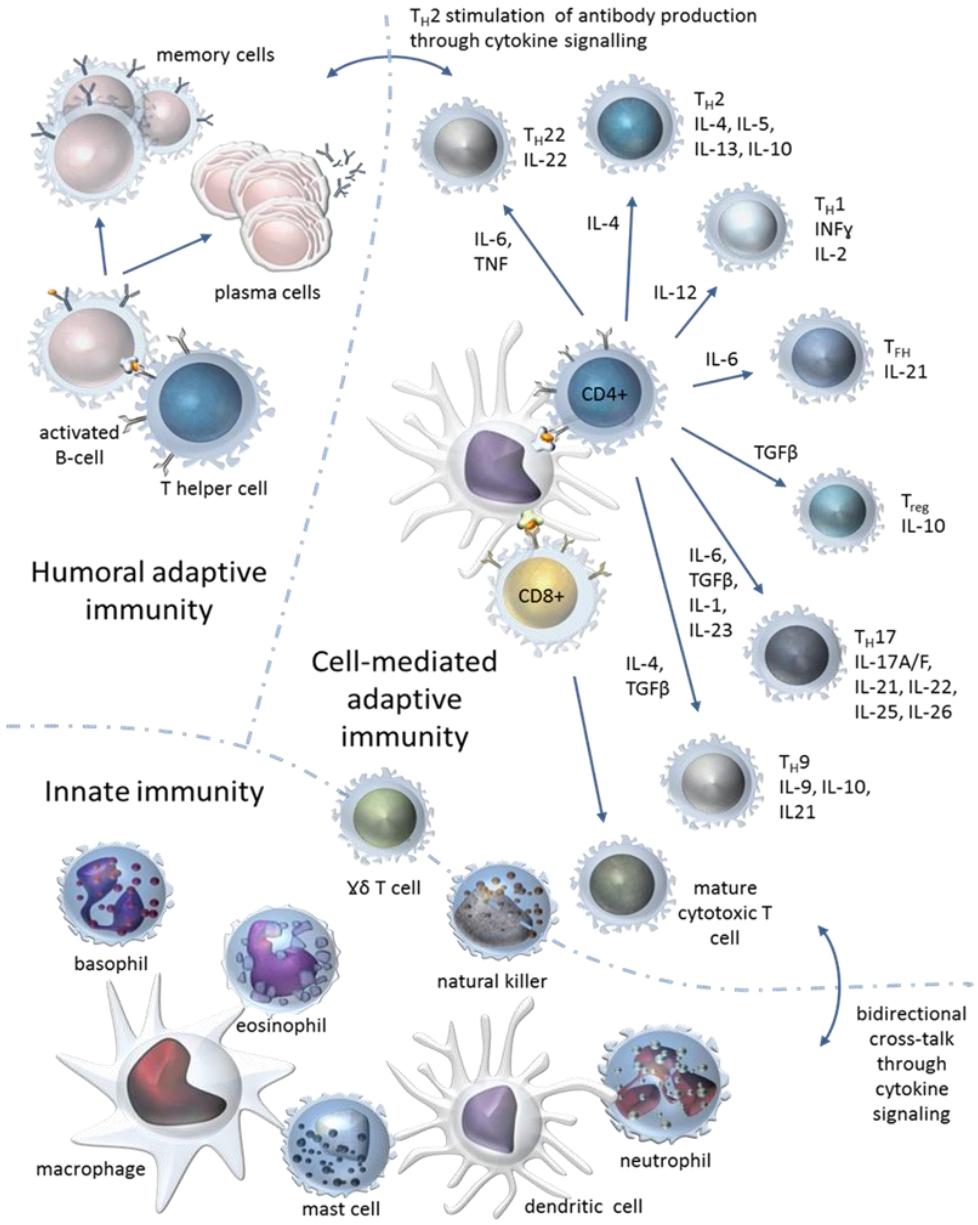


Figure 1.2 Cells of the innate and adaptive immune systems. The innate system is comprised of multiple anatomical and physiological barriers and compliment (not shown) along with numerous cells whose function is to respond quickly to infection, induce inflammation and/or activate adaptive immunity. The adaptive system is divided into two branches, humoral and cell-mediated. The humoral system is characterized by antibody production by B cells. Cell-mediated immunity encompasses T cell activities that regulate innate and humoral responses or that function against cancers and intracellular pathogens. T cells differentiate from CD4+ or CD8+ cells upon exposure to antigens displayed on MHC II or MHC I molecules, respectively, of antigen presenting cells (APC) . Differentiation of T cell is driven by cytokines resulting in T cell subset each producing its own unique effector cytokine profile. Cross-talk through cytokine signalling occurs between the innate and adaptive system and within the adaptive system.

The adaptive immune system targets specific antigens and thus takes time to develop. However, it exhibits immunological memory and will respond more rapidly upon subsequent exposure. The adaptive immune system has two arms: the humoral immune response and cell-mediated immunity. Humoral immunity typically acts against extracellular pathogenic agents and includes all activities related to antibody production and is largely mediated by B lymphocytes and T helper 2 cells. Cell-mediated immunity does not involve the generation of antibodies, but employs phagocytes and cytotoxic T lymphocytes, which primarily target intracellular pathogens.

T cells facilitate cell-mediated immunity through regulating immune responses or by directly attacking infected cells. T cells are classified based on their function and surface markers, with the majority being classified into two main types; CD4 and CD8, based on the types of glycoproteins expressed on their surfaces. The CD8+ T cells are called cytotoxic T lymphocytes; their role is to destroy any cell that threatens host integrity. They do so by recognizing antigens displayed by major histocompatibility complex (MHC) I molecules on host cells that are infected with viruses or bacteria. T cells that express CD4+ are called T helper lymphocytes and recognize antigens displayed on the MHC II molecules of antigen presenting cells. T helper cells are responsible for modulating both cell mediated and humoral responses through the production of signaling molecules called cytokines.

There are multiple subsets of T helper cells, each secreting a specific cytokine profile. These are described in greater detail elsewhere (Luckheeram et al., 2012), with the primary profiles summarized here. T helper 1 ($T_H1$) cells promote cell-mediated responses that eliminate intracellular pathogens and are most commonly associated with secretion of interferon ɣ (INFɣ) and interleukin 2 (IL-2)(Raphael et al., 2014). T helper 2 ($T_H2$) cells promote humoral responses that target extracellular parasites and play a major role in asthma and allergic reactions. Key effector cytokines for $T_H2$ responses include IL-4, IL-5, and IL-13 (Raphael et al., 2014). T helper 17 ($T_H17$) cells are responsible for enhancing anti-microbial responses at epithelial and mucosal barriers and produce the effector cytokines IL-17, IL-22, IL-21, IL-25 and IL-26. T helper 22 ($T_H22$) cells are associated immunopathology of skin diseases and produce predominantly IL-22 (Raphael et al., 2014). T helper 9 ($T_H9$) cells produce IL-9, IL-10 and IL-21 and are involved in promoting mucus production and activating mast cells and eosinophils (Kaplan et al., 2015; Raphael et al., 2014). Regulatory T cells ($T_{reg}$) are immune regulators that play an important role in immunological tolerance, and are defined by their expression of FoxP3 producing the main effector cytokines IL-10 and transforming growth factor beta (TGF-β). Lastly, follicular helper T cells ($T_{FH}$) reside in the B cell follicles and play a role in humoral immunity through interactions with B-lymphocytes and primarily produce IL-21(Luckheeram et al., 2012; Zhu and Paul, 2010).

### 1.5.2 Measuring Immune Responses

Multiple assays exist to measure both humoral and cell-mediated responses to antigens. To measure the magnitude of humoral responses, ELISA is commonly employed to determine both overall antibody titre, but also to examine isotypes of the sera generated, thus the class or subclass of antibody produced. There are several assays that can be employed to measure cellular immunity, or T cell responses. Older methods include delayed type hypersensitivity (DTH), chromium release assays, and limiting dilution assays (LDA) (Hobeika et al., 2005). Newer assays include intracellular cytokine assays, peptide: MHC tetramers and ELISPOT (Hobeika et al., 2005). Conventional assessment of immune responses has typically focused on the frequency, magnitude or quantity of the immune response, although quality of response is also a critical component (Doolan et al., 2014). Quality of antibody responses can be assessed be examining affinity, avidity, diversity and biological function and subclass, while quality in T cell response can be accessed via cell phenotype, differentiation state, cytokine profiles, T-cell receptor avidity and diversity (Doolan et al., 2014).

Almost all of the currently licenced vaccines rely on the induction of functional antibodies against the target pathogen (Doolan et al., 2014). There are no vaccines that are designed to specifically induce cellular immune responses (Doolan et al., 2014), although these responses play a critical role in the targeting and destruction of pathogen-infected cells or through the activation of i B cell expansion and differentiation, class switching, and affinity maturation of the responses (Sette and Rappuoli, 2010). Although RV can be used as a means to enhance the rate of vaccine design, one of the most challenging steps is to evaluate not only the response to antigen exposure, but the quality of that response and how it translates into vaccine functionality. Further, the identification of suitable antigens is only one step in vaccine design while vaccine development requires investments in vaccine formulation, proof of principal challenge studies, large scale clinical trials, patenting, licencing regulatory approval, and marketing.

## 1.6 Conclusion

Bovine respiratory disease is one of the principal health concerns for the North American beef production industry. Understanding the epidemiological aspect of viral and bacterial agents contributing to this condition is necessary to highlight strategies for its management. As a major bacterial agent of BRD, *M. haemolytica* is of particular interest for study. Comparative analysis between whole genome sequences of pathogenic and commensal serovars of this bacterium allows for the examination of virulence mechanisms that have not been phenotypically defined. This analysis can also be applied to identify novel antigens for vaccine development that were previously unknown in this

species. Antimicrobial are the primary means for prevention and treatment of bacterial agents involved in BRD in feedlot cattle and the development of widespread antimicrobial resistance could prove to be economically devastating to the cattle industry. The availability of efficacious vaccines against bacterial agents of BRD will aid in reducing dependencies on antimicrobial therapies to control these organisms in the feedlot setting. Reverse vaccinology offers an alternative strategy to conventional methodologies used for vaccine design against agents of BRD.

The intent of this project was to examine populations of pathogens associated with BRD cases collected from a wide range of geographical locations. *Mannheimia haemolytica* was targeted for whole genome sequencing and comparative genomic analysis of pathogenic and non-pathogenic serovars in an effort to identify antigen candidates that could be explored further for vaccine development. The immunoreactivity of the identified candidates was also to be evaluated. To pursue this, multiple objectives were defined, as outlined below.

**1.7 Hypothesis:**

Reverse vaccinology and comparative genomics of *M. haemolytica* isolates of varying virulence and a diverse geographic origin can be used to rapidly identify novel protein candidates for vaccine development.

**1.8 Objectives:**

1. To examine *M. haemolytica* collected from healthy cattle and those with BRD to better characterize populations associated with respiratory disease in feedlot cattle.
2. To examine BRD mortalities from feedlots in North America for the occurrence of viral and bacterial agents associated with disease and to characterize their antimicrobial resistance profiles and any associated mobile genetic elements conferring resistance genes.
3. To undertake comparative genomic analysis on 11 strains of *M. haemolytica* representing serotypes 1, 2 and 6 from healthy cattle or cattle that were morbid or succumbed to BRD in an effort to better understand the genes contributing to virulence in this species.
4. To use a RV approach to identify antigen candidates that may be components of a vaccine designed against *M. haemolytica* and develop a high throughput method to screen antigens for immunoreactivity.

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## 2 Chapter 2: Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease

Chapter 2 has been published in the Canadian journal of Veterinary Research.
Klima, C.L., Alexander, T.W., Hendrick, S., McAllister, T.A., 2014. Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. Can. J. Vet. Res. 78, 38-45.

This manuscript was drafted by Cassidy Klima with suggestions and comments from the collaborating authors. Experimental design, data collection and analysis were undertaken by Cassidy Klima, Dr. Alexander and Shaun Cook.

**2.1 Introduction**

Bovine respiratory disease (BRD) is a considerable health problem in cattle within confined feeding facilities in North America. In western Canada, 10% to 30% of all auction derived cattle are treated for BRD, with 5% to 10% of those expected to succumb to the disease (Booker et al., 2008). Typically manifesting as severe pneumonia, BRD is usually observed in cattle within 2 months of arrival at the feedlot (Gagea et al., 2006). Economic losses from the condition are primarily due to treatment costs and associated animal handling expenditures, reductions in carcass quality, and death (Snowder et al., 2007).

Causation of BRD is linked to environmental stressors that predispose cattle to viral and bacterial infection (Taylor et al., 2010) by various agents including bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus ($PI_3V$), bovine herpesvirus (BHV-1)/(BHV-4), *Pasteurella multocida, Mycoplasma bovis, Histophilus somni, and Mannheimia haemolytica. Mannheimia haemolytica* is the bacterium most commonly associated with the gross pathology of the acute fulminating bronchopneumonia, typically seen within the first few weeks that cattle are on feed (Confer, 2009). This, coupled with the systematic detection of *M. haemolytica* in BRD cases (Rice et al., 2007), has resulted in *M. haemolytica* being considered one of the predominant bacterial pathogens associated with respiratory disease in cattle.

Although recognized as a contributing factor to illness, *M. haemolytica* is also found as a commensal bacterium in the upper respiratory tract of healthy ruminants (Confer, 2009). Virulence is strongly associated with capsular serotype; serotypes 1 and 6 are most commonly associated with BRD (Rice et al., 2007). Extensive work focusing on the characterization of virulence factors in *M. haemolytica* (Highlander, 2001; Zecchinon et al., 2005) and the recent sequencing of the *M. haemolytica* genome (Gioia et al., 2006; Lawrence et al., 2010) has helped elucidate much of the genetic basis for its pathogenesis. However, most of these studies have focussed on serotype 1 isolates or are limited to only a few strains. Comparisons of epidemiological isolates from healthy cattle and those diagnosed with BRD may also provide important information on bacterial factors that contribute to the development of BRD. The objective of this study was to examine *M. haemolytica* collected from healthy cattle and those with BRD in an effort to develop a better understanding of the characteristics of *M. haemolytica* populations associated with respiratory disease in feedlot cattle.

## 2.2 Materials and methods

### 2.2.1 Bacterial Isolates

*Mannheimia haemolytica* used in this study were isolated as part of longitudinal surveillance program of commercial beef feedlots in western Canada (Klima et al., 2011). Samples were collected from the nasopharynx of beef cattle using commercially available deep-guarded culture swabs with a Cary-Blair agar reservoir for transport (BD Canada, Mississauga, Ontario) and processed for isolation of *Mannheimia haemolytica* as previously described (Klima et al., 2011). Randomly selected isolates that were collected from fall-placed calves with a known history of respiratory health were categorized into 2 groups: i) those isolated from healthy cattle (not treated for BRD while placed in feedlots, $n$ = 49) and ii) those isolated from cattle treated for BRD ($n$ = 41). Isolates from healthy cattle were collected when they arrived ($n$ = 27) or when they exited feedlots ($n$ = 22). Healthy cattle were placed in feedlots for a minimum of 60 days. *Mannheimia haemolytica* from cattle with BRD were isolated at the time of initial treatment. Trained animal health feedlot staff identified the calves as sick and then removed them from their pen. The rectal temperature of these calves was measured and if it was > 40°C, they were swabbed and treated as per the feedlot's protocol. All isolates were identified as *M. haemolytica* by biochemical and polymerase chain reaction (PCR) tests as previously described (Alexander et al., 2008).

### 2.2.2 Serotyping

Serotyping was performed using the rapid plate agglutination procedure as described by Frank and Wessman (Frank and Wessman, 1978). Rabbit antisera were raised against reference strains of *M. haemolytica* as previously described (Klima et al., 2011).

*Pulsed-field gel electrophoresis (PFGE)*

Molecular typing was performed by pulsed-field gel electrophoresis (PFGE) according to methods previously reported (Klima et al., 2010). Macro-restriction digest was conducted on all *M. haemolytica* isolates using *Sal*I restriction enzyme. The resulting PFGE patterns were assessed using BioNumerics V5.1 software (Applied Maths, Austin, Texas, USA). Dendrogram and minimum spanning tree (MST) analysis was based on similarity matrices generated from UPGMA clustering of Dice coefficient values. Optimization and position tolerance settings for dendrogram analysis were defined at 1.00% and 1.50%, respectively. Simpson's diversity indices were calculated using the online script tools available for BioNumerics V5.1 software. Bin size for MST was set to 3.0% and complexes were generated with a maximum neighbor distance of 2.

### 2.2.3 Sensititre

Antimicrobial susceptibility testing was performed by broth microdilution using a commercially available panel (Bovine/Porcine with Tulathromycin MIC Format, Sensititre; Trek Diagnostic Systems, Cleveland, Ohio, USA). A list of the antimicrobials utilized and the range of concentrations tested are presented in Table 2.1. In addition to these, sulphadimethoxine and trimethoprim/sulfamethoxazole were included in the panel , but at single breakpoint concentrations containing 256 µg/mL sulphadimethoxine and 2/38 µg/mL trimethoprim/sulfamethoxazole, respectively. Plates were read manually by visual assessment and assignment of growth was defined according to recommendations provided in the Clinical and Laboratory Standards Institute (CLSI) document M31-A3 (CLSI, 2008). At the time of experimentation, (CLSI) breakpoints were not available for clindamycin, neomycin, penicillin, tiamulin, sulphadimethoxine, trimethoprim/sulfamethoxazole, or tylosin tartrate. Therefore, susceptibility designations for these drugs were not assigned. Exceptions to this are cases in which isolates exhibited a high MIC for neomycin (MIC 32 µg/mL) or penicillin (MIC 8 µg/mL) and harbored the corresponding antimicrobial resistance gene determinant, *aphA*-1 and $bla_{\text{ROB-1}}$, respectively. In these instances the isolates were classified as neomycin- or penicillin-resistant. The Fisher's exact test was used to compare differences in the prevalence of antimicrobial resistant *M. haemolytica* isolated from treated and untreated cattle.

Table 2.1 Frequency distribution of MICs of 88 *Mannheimia haemolytica* collected from the nasopharynx of feedlot cattle

| Antibiotic | Number of isolates (%) showing a MIC (µg/ml) of:[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 |
| Ampicillin[b] | | 83 (94.3) | - | - | - | - | - | 5 (5.7) | | |
| Clindamycin[c] | | | - | - | 3 (3.4) | 33 (37.5) | 40 (45.5) | 12 (13.6) | | |
| Chlortetracycline[b] | | | 49 (55.7) | 23 (26.1) | 13 (14.8) | 3 (3.4) | | | | |
| Danofloxacin[b] | 88 (100) | - | - | - | | | | | | |
| Enrofloxacin[b] | 88 (100) | - | - | - | - | | | | | |
| Florfenicol[b] | | 5 (5.7) | 73 (83.0) | 10 (11.4) | - | - | - | | | |
| Gentamicin[b] | | | | 41 (46.6) | 46 (52.3) | 1 (1.1) | - | - | | |
| Neomycin[c] | | | | | | 54 (61.4) | 21 (23.9) | - | 13 (14.8)* | |
| Oxytetracycline[b] | | | 51 (58.0) | 20 (22.7) | - | 1 (1.1) | 16 (18.2) | | | |
| Penicillin[c] | 67 (76.1) | 14 (15.9) | 2 (2.3) | - | - | - | 5 (5.7)* | | | |
| Spectinomycin[b] | | | | | | | - | 48 (54.5) | 39 (44.3) | 1 (1.1) |
| Tiamulin[c] | | | - | - | - | - | 22 (25.0) | 63 (71.6) | 3 (3.4) | |
| Tilmicosin[b] | | | | | | 78 (88.6) | 5 (5.7) | - | 5 (5.7) | |
| Ceftiofur[b] | | 88 (100) | - | - | - | - | - | | | |
| Tulathromycin[b] | | | | | 49 (55.7) | 33 (37.5) | 6 (6.8) | - | - | - |
| Tylosin tartrate[c] | | | - | - | - | - | - | 1 (1.1) | 87 (98.9) | |

[a]Dotted and full lines indicate breakpoints for intermediately resistant and resistant, respectively. Blanks indicate that isolates were not tested at the concentration specified. Values presented at lowest concentrations tested include the number of isolates with MIC at that value but also those isolates with an MIC of 0 for that drug.

[b]Breakpoints determined from CLSI guidelines (CLSI, 2008)

[c]Breakpoint not available

*Resistance breakpoint defined by authors

### 2.2.4 PCR

Polymerase chain reaction screening for antimicrobial resistance genes was performed on *M. haemolytica* isolates exhibiting antimicrobial resistant phenotypes. A list of the resistance genes evaluated, primer sets used (Chen et al., 2007; Kadlec et al., 2011; Klima et al., 2011; Ng et al., 2001) and the resistant phenotypes targeted is presented in Table 2.2. Screening for virulence-related genes was conducted on all isolates used in the study. A list of the virulence genes evaluated and primer sets used (Lo et al., 2006) is presented in Table 2.3.

Table 2.2 Primers used to screen for antimicrobial resistance genes in *Mannheimia haemolytica* isolated from feedlot cattle

| Phenotype targeted[a] | Resistance gene | PCR primer sequence 5'-3' | Amplicon size | Annealing temp (°C) | Primer reference |
|---|---|---|---|---|---|
| $TET^R$ | *tet(*H) | ATACTGCTGATCACCGT | 1076 | 60 | 11 |
| | | TCCCAATAAGCGACGCT | | | |
| $AMP^R$, $PEN^R$ | $bla_{ROB-1}$ | AATAACCCTTGCCCCAATTC | 685 | 60 | 11 |
| | | TCGCTTATCAGGTGTGCTTG | | | |
| $TIL^R$ | *erm(*X) | GAGATCGGRCCAGGAAGC | 488 | 58 | 17 |
| | | GTGTGCACCATCGCCTGA | | | |
| | *erm*(42) | GGGTGAAAAGGGCGTTTATT | 1,254 | 60 | 18 |
| | | ACGTTGCACTTGGTTTGACA | | | |
| | *msr*(E)-*mph*(E) | TACCGGAACAACGTGATTGA | 3,277 | 60 | 18 |
| | | GAAGGGTTACGCCAGTACCA | | | |
| $NEO^R$ | *aphA-1* | TTATGCCTCTTCCGACCATC | 489 | 54 | 16 |
| | | GAGAAAACTCACCGAGGCAG | | | |

[a] $TET^R$, tetracycline resistant; $AMP^R$, ampicillin resistant; $PEN^R$, penicillin resistant; $TIL^R$, tilmicosin resistant; $NEO^R$, neomycin resistant

All PCR reaction mixtures (20 µL) contained (final concentrations): 1 × HotStarTaq *Plus*Master Mix (Qiagen Canada, Toronto, Ontario), 0.2 µM primer and 1 × CoralLoad Concentrate (Qiagen Canada). Colony lysate was used for template DNA and generated by incubating single colonies of bacteria in 50 µL of Tris EDTA buffer (pH 7.4) at 98°C for 5 min. Lysate was centrifuged at 13 000 × *g* for 5 min, after which 2 µL of supernatant was added to the PCR as template DNA. Polymerase chain reaction amplifications were performed using an Eppendorf Mastercycler (Eppendorf Canada, Mississauga, Ontario) with the following conditions: 95°C for 5 min; 30 cycles of 94°C for 30 s, respective annealing temperatures (refer to Tables 2.2 and 2.3) for 1 min, 72°C for 1 min and 72°C for 8 min. For *erm*(42) and *msr*(E)-*mph*(E) PCRs, the extension time was increased to 1.5 min to compensate for larger

products. A touchdown assay, as previously described (Chen et al., 2007), was used for *erm*(X) gene screening.

Table 2.3 Primers used to screen for virulence genes in *Mannheimia haemolytica* isolated from feedlot cattle[a]

| Resistance gene | Virulence-related genes | PCR primer sequence 5'-3' | Amplicon size | Annealing temp (°C) |
|---|---|---|---|---|
| *gcp* | O-sialoglycoprotease | CGCCCCTTTTGGTTTTCTAA | 420 | 58 |
| | | GTAAATGCCCTTCCATATGG | | |
| *gs60* | Outer-membrane lipoprotein | GCACATTATATTCTATTGAG | 429 | 55 |
| | | AGGCATACTCTAACTTTTGC | | |
| *tbpB* | Transferring-binding protein B | CTACTTGCTGCTTGTTCCTC | 137 | 60 |
| | | AGAACCGCTTACTGTACGTC | | |
| *lktC* | Leukotoxin | GGAAACATTACTTGGCTATGG | 440 | 58 |
| | | TGTTGCCAGCTCTTCTTGATA | | |
| *nmaA* | UDP-N-acetyl-D-glucosamine-2-epimerase | CTGTAGAAGCCGGAACAGTA | 129 | 60 |
| | | CATCGCCATAAGGGTTGTGA | | |
| *adh* | Putative adhesion | CTGCAAGTAAGGCAACATTG | 150 | 58 |
| | | GAATCCGCACCAATAGCAAT | | |

[a]From reference 19.

## 2.3 Results

### 2.3.1 Serotype

All isolates, 49 collected from healthy animals located in 2 feedlots and 41 collected from BRD cases from 3 feedlots, typed as either serotype 1 (S1), 2 (S2), or 6 (S6) (Table 2.4). Over 75% of isolates collected from healthy animals at either arrival or exit were identified as S2, whereas 90% of isolates from cattle with BRD were either S1 or S6.

Table 2.4 Serotypes of *Mannheimia haemolytica* collected from the nasopharynx of healthy cattle or cattle treated for bovine respiratory disease (BRD)

| | | Serotype (%) | | |
|---|---|---|---|---|
| Cattle group | No. of isolates | 1 | 2 | 6 |
| Healthy | 49 | 7 (14.3) | 37 (75.5) | 5 (10.2) |
| BRD-treated | 41 | 29 (70.7) | 4 (9.7) | 8 (19.5) |

### 2.3.2 Pulsed-field gel electrophoresis

Dendrogram analysis of the restriction patterns from all isolates revealed considerable diversity, with 80 unique pulsotypes (data not shown). Two main clusters comprised of 51 and 39 isolates each were identified and exhibited a similarity of 39%. Diversity within each main cluster was high, as indicated by a Simpson's diversity index of 92.4% for the larger and 92.3% for the smaller cluster. A minimum spanning tree generated from the PFGE similarity matrices reproduced the two main clusters described (Figure 2.1). The first cluster (*n*= 51) was comprised largely of S1 and S6 isolates, while the second cluster (*n* = 39) was comprised solely of S2 isolates.

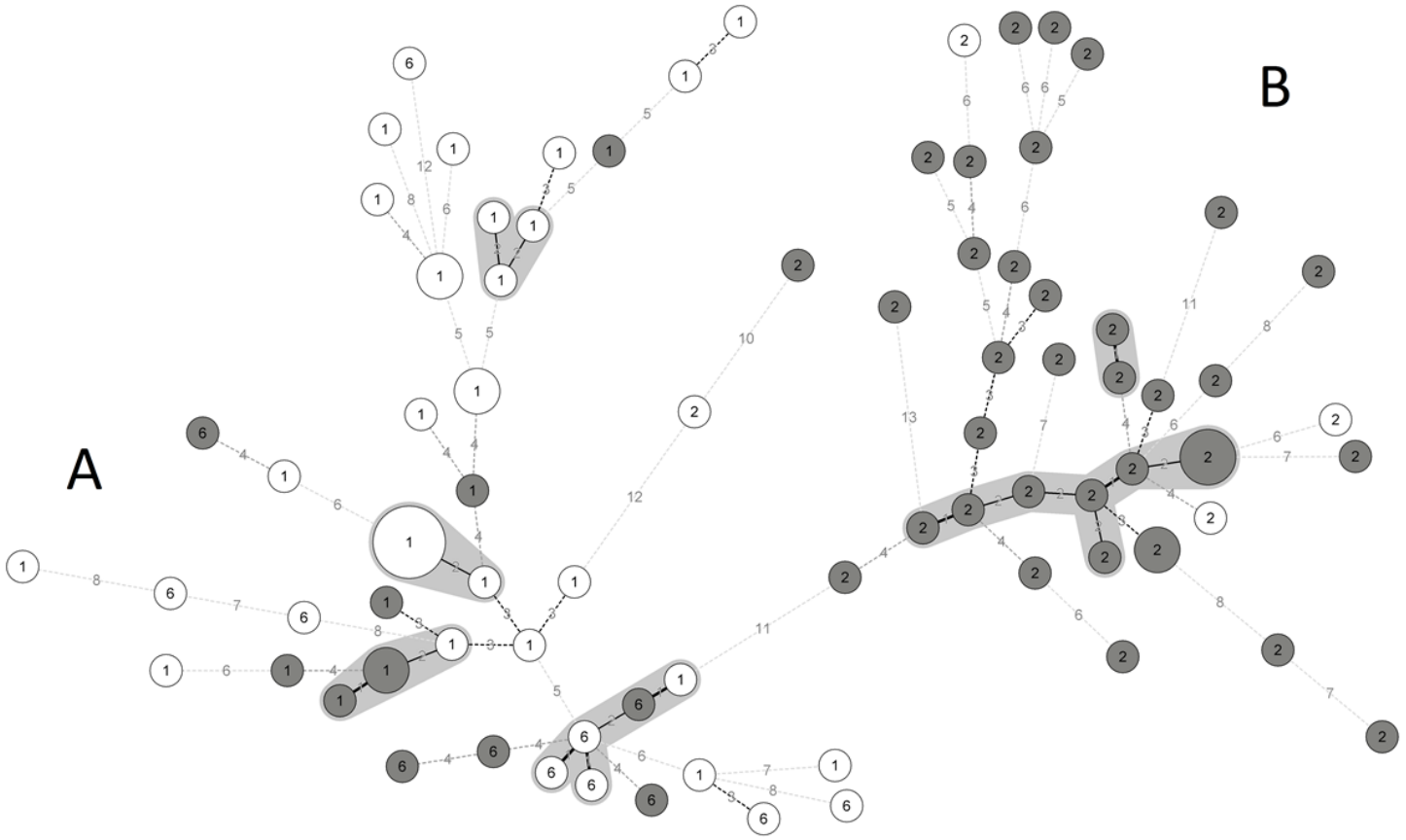
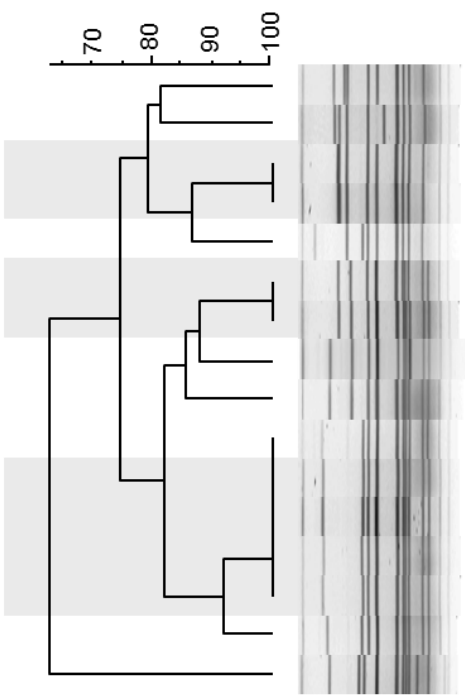



Figure 2.1 Minimum spanning tree based on similarity matrix generated from pulsed-field profiles of *Mannheimia haemolytica* isolates collected from feedlot cattle. Bin size of 3.0% with distance indicated. Complexes were generated with a maximum distance of 2 (shown in shaded area around groups of isolates) and circle size is proportional to member count. Colors inside of circles indicate if the isolate came from an animal that was healthy (grey) or had BRD (white). Numbers inside of circles refer to serotype. Two main groups labeled A and B, dominate the tree. Group A is comprised of mostly S1 and S6 isolates and Group B is comprised completely of S2 isolates.

### 2.3.3 Antimicrobial Susceptibility

All *M. haemolytica* isolates grew in 256 µg/mL sulphadimethoxine, but failed to grow in 2/38 µg/mL trimethoprim/sulfamethoxazole. The MICs for the 16 antimicrobials tested are reported in Table 2.1. Overall, resistance to at least one antimicrobial was observed in 16 (18%) isolates. Resistance to oxytetracycline was most common (18%), followed by neomycin (14.8%). Five tetracycline–neomycin resistant isolates also exhibited resistance to ampicillin, penicillin, and tilmicosin. There was a higher prevalence ($P < 0.001$) of antimicrobial resistance observed for isolates obtained from cattle with BRD (37%) as compared to those that were healthy (2%).

Of the 16 antimicrobial-resistant isolates detected, only one was recovered from a healthy animal and the remaining were isolated from cattle exhibiting BRD (Figure 2.2). Most of these isolates exhibited multi-drug resistance, with 13 isolates being resistant to at least 2 antimicrobials. All of the isolates exhibiting antimicrobial resistance phenotypes were S1. There were 3 cases in which isolates with the same pulsotype, antimicrobial resistance phenotype, and resistance genes were obtained from different cattle. This was observed in cases in which animals were located in different feedlots, located in the same feedlot but in different pens, and located in the same feedlot and pen.




| Isolate | Pen | Feedlot | Resistance Phenotype | AMR Gene |
|---|---|---|---|---|
| H28 | 77 | 3 | Tet, | *tetH* |
| H7 | B1 | 1 | Tet, Neo | *tet*H, *aphA*-1 |
| H16 | D8 | 2 | Tet ,Neo | *tet*H, *aphA*-1 |
| H30 | A7 | 1 | Tet, Neo | *tet*H, *aphA*-1 |
| H36 | E6 | 1 | Amp, Pen, Tet, Neo, Til | *tet*H, $bla_{ROB-1}$, *aphA*-1 |
| H32 | A10 | 1 | Tet, Neo | *tet*H, *aphA*-1 |
| H6 | B1 | 1 | Tet Neo | *tet*H, *aphA*-1 |
| 611[a] | N/A | 4 | Tet, Neo | *tet*H, *aphA*-1 |
| H51 | A9 | 3 | Tet, Neo | *tet*H, *aphA*-1 |
| H18 | D8 | 2 | Tet, Neo | *tet*H, *aphA*-1 |
| H25 | 77 | 3 | Amp, Pen, Tet, Neo, Til | *tet*H, $bla_{ROB-1}$, *aphA*-1 |
| H26 | 77 | 3 | Amp, Pen, Tet, Neo, Til | *tet*H, $bla_{ROB-1}$, *aphA*-1 |
| H27 | 77 | 3 | Amp, Pen, Tet, Neo, Til | *tet*H, $bla_{ROB-1}$, *aphA*-1 |
| H29 | 77 | 3 | Amp, Pen, Tet, Neo, Til | *tet*H, $bla_{ROB-1}$, *aphA*-1 |
| H19 | D9 | 2 | Tet | *tet*H |
| H17 | C10 | 2 | Tet | *tet*H |

Figure 2.2 Dendrogram analysis of pulsed-field profiles from *Mannheimia haemolytica* isolates expressing antimicrobial-resistant phenotypes. The dendrogram was created using UPGMA clustering of Dice coefficient values. Similarity matrix was based on band-matching analysis, optimization and tolerance settings of 1.0% and 1.5%, respectively.

[a] The only isolate collected from a healthy animal to express an antimicrobial resistance phenotype. Tet — tetracycline resistant; Amp — ampicillin resistant; Pen — penicillin resistant; Til — tilmicosin resistant; Neo — neomycin resistant; AMR — antimicrobial resistance.

### 2.3.4 Resistance genes

Polymerase chain reaction screening showed agreement between antimicrobial resistant phenotypes and the presence of an associated antimicrobial resistance gene. Oxytetracycline resistance corresponded to the presence of *tet*(H), neomycin resistance to the presence of *aphA*-1, and ampicillin/penicillin resistance to the presence of $bla_{ROB-1}$ (Figure 2.2). Neither *erm*(X), *erm*(42) nor the *msr*(E)-*mph*(E) genes that confer resistance tilmicosin were identified.

### 2.3.5 Virulence related genes

Polymerase chain reaction screening for the virulence-related genes leukotoxin C *(lktC),* a putative adhesin *(ahs),* an outer- membrane lipoprotein *(gs60),* an O-sialoglycoprotease*(gcp),* transferring-binding protein B *(tbpB),* and UDP-N-acetyl-D-glucosamine-2-epimerase*(nmaA)* revealed the presence all 6 genes in S1 and S6 isolates regardless of whether the isolates were obtained from healthy cattle or cattle with BRD. The *tbpB* and *nmaA* gene were not detected in any of the S2 isolates, whereas *lktC, ahs, gs60,* and *gcp,* were present.

## 2.4 Discussion

### 2.4.1 Serotype

Of the 12 serotypes currently described, S1 and S6 are thought to account for most *M. haemolytica*-associated BRD worldwide (Rice et al., 2007), with S1 recovered most frequently from pneumonic lesions in cattle (Lo et al., 2006). The findings here are consistent with this position, as 92% of the isolates collected from cattle with BRD were either S1 or S6, with the majority being S1. Serotype 2 is commonly recognized as a commensal organism in cattle (Lawrence et al., 2010), but has been identified as a causative agent of respiratory disease in sheep (Davies et al., 2001). Here, the recovery of S2 from healthy cattle was close to 8 times higher than that from cattle with BRD.

Of the cattle exhibiting BRD, S1, S2, and S6 serotypes accounted for approximately 70%, 10%, and 20% of the isolates obtained. This distribution agrees with other reports in which *M. haemolytica* has been isolated from cattle exhibiting BRD, in the western (60% S1, 7% S2, and 26% S6) (Al-Ghamdi et al., 2000) and mid-western USA (60% S1, 10% S2, and 27% S6) (Purdy et al., 1997). It is unclear why, given the broad sampling range and period between investigations, a similarity in the distribution of serotypes should be observed between studies. Although the prevalence of S2 in nasal samples has been shown to decrease after transportation of cattle from farms to feedlots (Frank and Smith, 1983), S2 has been shown to account for 74% of *M. haemolytica* colonizing the nasopharynx of feedlot cattle (Klima et al., 2011). Therefore, there is a clear shift in serotype populations, depending on animal health.

Although evidence suggests serotype-related differences exist in *M. haemolytica* host specificity and virulence (Davies et al., 2001), this relationship is not definitive as in 4 instances S2 isolates were recovered from cattle with BRD. However, these isolates were not closely related and it is interesting to note that the pulsotype for one of the S2 isolates grouped more closely to S1 and S6 isolates. Although serotype conversion has been investigated in *M. haemolytica* with both genotypic and phenotypic variability described in S1 and S2 isolates upon successive subculture (Villard et al., 2008), direct evidence for serotype switching has not been documented in this species. It is unlikely, based upon the dissimilarity in pulsotype observed between the serotypes examined, that serotype conversion is playing a role in these populations. It is possible that S2 isolates collected from diseased animals are a subset of the ubiquitous populations and are adapted in a way that makes them capable of extensive proliferation in immune-compromised bovine hosts.

**2.4.2 Polymerase chain reaction of virulence related genes**

In this study we used PCR to screen for the presence of 6 genes previously shown to be related to virulence in *M. haemolytica*. The results from this analysis show a consistent relationship between isolate serotype and virulence gene profile. With the described primer sets, *tbpB,* which encodes transferring-binding protein B that is implicated in iron acquisition, and *nmaA,* which encodes UDP-N-acetyl-D-glucosamine-2-epimerase that is involved in capsule biosynthesis, were detected in all S1 and S6 isolates, while they were not detected in S2 isolates. This suggests that these genes are either absent in S2 isolates or they have divergent sequences that were not amplifiable by the PCR assays. Additionally, use of PCR to target virulence genes specific to serotypes of *M. haemolytica* could circumvent the need for serum based typing methods. Further work would be needed to determine if the genes utilized in this study are useful for such a purpose, but these preliminary data suggest that the presence of *nmaA* could be useful in differentiating between S2 and either S1 or S6 strains of *M. haemolytica*. Further investigations into targets such as these could also provide potential candidates for serotype-specific vaccine development against *M. haemolytica*.

**2.4.3 Pulsed-field gel electrophoresis**

Analysis of PFGE patterns revealed levels of genetic diversity similar to what has been previously reported for *M. haemolytica* collected from the nasopharynx of beef cattle (Klima et al., 2011). Two main groups were observed to cluster by serotype and to a lesser degree by the health status of the host from which the isolates were obtained. There were 3 instances in which highly related isolates (100% similarity) were recovered from different cattle. Clones observed in cattle within different pens and feedlots likely reflect the nature of the beef industry in which animals are mixed at auction marts and

then separated into groups at feedlots. Based on these data, it is difficult to determine the stage at which these isolates spread among cattle, be it on-farm, at auction, during transport, or once inside the feedlot. We have previous evidence of clonal spread of *M. haemolytica* in the general feedlot population (Klima et al., 2011), but a striking observation in the present study was that all cases of clones were S1 isolates collected from cattle exhibiting BRD. This suggests that strain virulence may be an important factor in predisposition to *M. haemolytica*-related BRD.

**2.4.4 Susceptibility testing**

*Mannheimia haemolytica* isolates exhibited a low level of antimicrobial resistance (18%), consistent with previous reports that have examined antimicrobial resistance in *M. haemolytica* isolated from beef cattle (Catry et al., 2005; Hendriksen et al., 2008; Klima et al., 2011). The most common resistance phenotype observed was to oxytetracycline, not unexpected as tetracyclines are frequently used in western Canadian feedlots, typically as a feed additive for the prevention of liver abscesses or as a long-acting injectable for metaphylactic and therapeutic use.

The use of macrolide drugs in both human and veterinary medicine made the detection of tilmicosin resistance here a source of interest. A reduction in the efficacy of macrolide drugs holds consequence not only for human health but also for BRD therapy as both tulathromycin and tilmicosin are used for the treatment of respiratory infection in cattle (Klima et al., 2011). Of the isolates examined, 5 were resistant to tilmicosin, while none were resistant to tulathromycin. Tilmicosin resistance has been detected infrequently in *M. haemolytica* (Catry et al., 2005; Hendriksen et al., 2008; Klima et al., 2011; Watts et al., 1994; Welsh et al., 2004), but recent characterization of the macrolide resistance genes *erm*(42), *msr*(E) and *mph*(E) that confer resistance to tulathromycin in *P. multocida* and *M. haemolytica* (Desmolaize et al., 2011), suggests that there is potential for increased levels of macrolide resistance in *M. haemolytica* populations in the future.

Although the overall antimicrobial resistance levels described here were low, higher levels of resistance were observed in isolates collected from BRD cattle than from healthy animals, with all resistant isolates being S1. This suggests that antimicrobial resistance provides an advantage to the dominant serotype implicated in BRD. However, reasoning for this is complicated by the fact that the second most prevalent serotype in cattle exhibiting BRD, S6, exhibited no resistance to the antimicrobials tested. If antimicrobial therapy is driving resistance development in disease-related populations then the presence of antimicrobial resistance in a portion of the S6 isolates collected from BRD cattle would have been anticipated.

There is substantial under-reporting of serotype information in most of the antimicrobial resistance surveys with *M. haemolytica*. Consequently only one record was obtained that reports quinolone resistance in S6 *M. haemolytica*, (Katsuda et al., 2009). This occurred in a group of isolates of identical genetic profile, indicating that spontaneous mutation occurred in a single population resulting in clonal spread of the phenotype. It is possible that S6 isolates are less prone towards the uptake of exogenous DNA and/or transferable genetic elements. However, the genetic elements required for competence have been detected in S1 and S2 isolates (Gioia et al., 2006; Lawrence et al., 2010) and appear to be conserved across *Pasteurellaceae* (Kuhnert and Christensen, 2008). There is also the possibility that S6 infection varies in presentation resulting in fewer S6 cases being treated with antimicrobials.

**2.5 Conclusion**

In conclusion, clear differences were observed between populations of *M. haemolytica* collected from healthy versus cattle exhibiting the symptoms of BRD. The most notable were the higher proportion of isolates from BRD cattle that exhibited antimicrobial resistance profiles, and the shift in prevalence from S2 isolates in healthy cattle to S1 and S6 isolates in cattle treated for BRD. Although difficult to explain, all cases of clonal relatedness were observed in isolates collected from cattle that had BRD. This may highlight the importance of strain virulence in bacterial-associated BRD and indicate the potential for spread and dissemination of multidrug-resistant *M. haemolytica* among beef cattle.

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# 3 Chapter 3: Pathogens of Bovine Respiratory Disease in North American Feedlots Conferring Multidrug Resistance via Integrative Conjugative Elements

Chapter 3 has been published in the Journal of Clinical Microbiology.

Klima, C.L., Zaheer, R., Cook, S.R., Booker, C.W., Hendrick, S., Alexander, T.W., McAllister, T.A., 2014b. Pathogens of bovine respiratory disease in North American feedlots conferring multidrug resistance via integrative conjugative elements. J. Clin. Microbiol. 52, 438-448.

This manuscript was drafted by Cassidy Klima with suggestions and comments from the collaborating authors. Experimental design, data collection and analysis were undertaken by Cassidy Klima, Dr. Rahat Zaheer and Dr. Tim McAllister.

**3.1 Introduction**

In newly received feeder calves, shipping fever or Bovine Respiratory Disease (BRD) is the leading cause of death and illness, contributing to millions of dollars in losses through treatment costs, reduced meat yield and mortalities (Larson and Step, 2012; Snowder et al., 2007). Stressors as a result of weaning, marketing and transport predispose calves to viral infection that in turn can precipitate bacterial pathogenesis in the lower respiratory track (Rice et al., 2007). Multiple viral and bacterial agents are implicated in BRD, including bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine herpesvirus 1 (BHV-1), parainfluenza 3 virus ($PI_3V$), *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pasteurella multocida* and *Histophilus somni*. The term bovine respiratory disease can cover a range of pneumonic illness, from acute fatal respiratory disease to chronic or prolonged intractable respiratory disease (Booker et al., 2008). Although not absolute, acute fibrinous pneumonia or acute fatal pneumonia is most commonly associated with *M. haemolytica,* while chronic caseonecrotic pneumonia is typically associated with *M. bovis* (Gagea et al., 2006; Woolums, 2010).

To date, vaccination has shown inconsistent efficacy against *M. haemolytica*, *P. multocida* and *H. somni* infection in feedlot cattle (Larson and Step, 2012). As a result, antimicrobials are often the primary management practice used to reduce the incidence of BRD in feedlot cattle. In western Canada, 20-50% of newly arrived feedlot placements receive injectable metaphylactic antimicrobials for BRD prevention (Checkley et al., 2010) while 75% of USA feedlots with capacities of over 8000 head use injectable antimicrobials at arrival (USDA, 2012)

As the largest user of antimicrobials worldwide (Silbergeld et al., 2008), the agricultural sector is a potential driving force for evolution, persistence and spread of antimicrobial resistance traits (Call et al., 2008), both in targeted pathogens and commensals of livestock (Catry et al., 2003). The use of antimicrobials for growth promotion and metaphylaxis raises concerns over the extent these practices might contribute to antimicrobial resistance (AMR) in zoonotic bacterial pathogens (McEwen and Fedorka-Cray, 2002) and reduce the efficacy of antimicrobials employed to control infectious disease in cattle.

Bacteria may be intrinsically resistant or acquire resistance to antimicrobial agents either by *de novo* mutation in their genomes or through the acquisition of additional genes via horizontal gene transfer through conjugation, transformation or transduction. Horizontal gene transfer through conjugation requires a multi-protein apparatus, typically synthesized by the donor strain that physically connects the donor and recipient (Wozniak and Waldor, 2010), enabling transfer of one or multiple genes. Typically conjugation systems employ plasmids or plasmid-derived DNA but, recently

chromosome-borne mobile genetic elements referred to as integrative conjugative elements (ICEs) have been identified (Wozniak and Waldor, 2010). These elements commonly harbour clusters of accessory genes, including multidrug resistance cassettes accumulated through recombination. Once generated, multidrug resistant elements can establish and persist in bacterial populations as the result of the use of a single antimicrobial that co-selects for the entire element.

The development of widespread resistance in BRD pathogens would be economically devastating to the cattle industry (Watts and Sweeney, 2010). To ensure prudent antimicrobial use, surveillance of antimicrobial resistance in bacterial agents of BRD is required if therapeutic efficacy is to be maintained. Likewise, it is important to monitor the development of cross resistance to antimicrobials from drug classes of importance to human medicine.

The objective of this study was to examine BRD mortalities from feedlots in North America for the occurrence of viral and bacterial agents associated with disease and to characterize their antimicrobial resistance profiles.

**3.2 Materials and Methods**

**3.2.1 Sample collection**

Nasopharyngeal swab and lung tissue samples were collected at post-mortem necropsy from 68 BRD mortalities presenting acute fibrinous pneumonia in feedlots within Alberta (*n* = 42), Texas (*n* = 6) and Nebraska (*n* = 20). Lung tissue samples consisted of a 4 $cm^3$ section excised from the perimeter of a pneumonic lesion, stored and transported in Cary-Blair media (BD Canada Inc., Mississauga, ON). Nasopharyngeal samples were collected using commercially available deep-guarded culture swabs with a Cary-Blair agar reservoir (BD Canada Inc., Mississauga, ON). Samples were stored at 4 °C for a period no longer than ten days prior to processing for bacterial isolation.

*Bacterial isolation of M. haemolytica, P. multocida and H. somni*

For each lung tissue sample, approximately 2 $cm^3$ was excised with a sterile scalpel and homogenized with 10 mL of sterile Brain Heart Infusion (BHI; BD Canada Inc., Mississauga, ON) in a stomacher (Stomacher 400 Circulator, Seward Laboratory Systems Inc., USA) for 30 s. A 500 µL aliquot of the stomacher suspension was serially diluted 1:10, with $10^{-1}$ and $10^{-2}$ dilutions and cultured onto Tryptic Soy Agar (TSA; BD Canada Inc., Mississauga, ON) plates containing 5% blood overnight at 37°C. An aliquot of the lung tissue stomacher suspension was stored in 20% glycerol at -80°C and later processed for the isolation of *H. somni*. For this, approximately 10 µL of the frozen stocks were used to inoculate 900 µL of BHI broth. A 100 µL aliquot of the resulting suspension was plated onto TSA plates containing 5% blood and incubated for 48 h at 37°C under a 5% $CO_2$ atmosphere.

Colonies displaying morphology indicative of *M. haemolytica* (i.e. white-grey, round, medium-sized, non-mucoid, exhibiting β-haemolysis) were confirmed as previously described (Alexander et al., 2008). Identity of colonies displaying morphology of *P. multocida* (translucent, greyish in colour, and mucoid in consistency) and *H. somni* (yellowish hue, haemolytic), were confirmed by PCR using HotStarTaq *Plus* Master Mix (Qiagen Canada Inc., Toronto, ON) according to manufacturer's specifications with primers and annealing conditions as described (Table 3.1). Colonies exhibiting morphologies not indicative of *M. haemolytica, P. multocida* or *H. somni* were co-isolated and genus/species identified based on DNA sequencing (Eurofins MWG Operon, Huntsville, AL) and BLAST analysis of 16S rRNA genes.

Table 3.1 PCR primers and annealing conditions used for bacterial species confirmation, virus detection, antimicrobial resistance gene screening and detection of integrative conjugative elements (ICEs) associated genes from ICE*Pmu1*

| PCR target /phenotype | Primer name (F/ R)[a] | PCR primer sequence 5'-3' | Amplicon size | Annealing temp (°C) | Primer reference |
|---|---|---|---|---|---|
| | | ***Bacteria species confirmation PCR*** | | | |
| *Mannheimia* sp. | MHlkt-int_F / MHlkt-int_R | GTCCCTGTGTTTTCATTATAAG/CACTCGATAATTATTCTAAATTAG | 385 | 58 | 45 |
| *Histophilus somni* | HS453_F / HS860_R | GAAGGCGATTAGTTTAAGAG/TTCGGGCACCAAGTRTTCA | 400 | 55 | 46 |
| *Pasteurella multocida* | PM23_F / PM23_R | GGCTGGGAAGCCAAATCAAAG/CGAGGGACTACAATTACTGTAA | 1432 | 52 | 47 |
| *Mycoplasma bovis* | PMB996-F / PMB1066-R | TCAAGGAACCCCACCAGAT/AGGCAAAGTCATTTCTAGGTGCAA | 71 | 60 | 17 |
| | M*bovis* probe | FAM-TGGCAAACTTACCTATCGGTGACCCT-TAMRA | - | - | |
| 16S rRNA | 27F/1492R | AGAGTTTGATCCTGGCTCAG/ GGTTACCTTGTTACGACTT | 1450 | 58 | 48 |
| | | ***Virus detection*** | | | |
| BVDV (ssRNA positive-strand) | [b]RT_BVDVF / RT_ BVDVR | AGCGAAGGCCGAAAAGAGGC/CAACTCCATGTGCCATGTACAG | 312 | 58 | *This work* |
| $PI_3V$ (ssRNA negative-strand) | [b]RT_$PI_3$VF / RT_ $P_{I3}$VR | CATAAGTGATCTAGATGATGATCC/TTCATCTAGAATCTGAACTACTCC | 460 | 55 | *This work* |
| | [c]N_$PI_3$VF / N_$PI_3$VR | TGTCTTCCACTAGATAGAGGGATAAAATT/GCAATGATAACAATGCCATGGA | 111 | 60 | 49 |
| BRSV (ssRNA negative-strand) | [b]RT_BRSVF / RT_ BRSVR | CAAACTAAATGACACTTTCAACAAG/CATTTCATTCCTTAGTACATTGTTG | 566 | 54 | *This work* |
| | [c]N_BRSVF / N_BRSVR | CGTAGTACAGGTGACAACATTG/ACCAAAGCAGCAACACATAGCAC | 422 | 63 | *This work* |
| BHV-1 (dsDNA) | BHV-1F / BHV-1R | TCGAGCGGCAAGAGCACAAG/GGAAATCTTCGTGGCCCAGATG | 662 | 62 | *This work* |
| | [c]N_BHV-1F / N_BHV-1R | TGAGGCCTATGTATGGGCAGTT/GGACACAACAAACAATGCGG | 422 | 60 | *This work* |
| | | ***Antimicrobial resistance phenotype*** | | | |
| *tet*(H), *tetR* / $CTET^R$, $OXYT^R$ | *tet(*H)_F / *tet*(H)_R | ATACTGCTGATCACCGT/TCCCAATAAGCGACGCT | 1076 | 60 | 18 |
| | ICE *tetR*_F / ICE *tetR*_R | CGGCTTGGGTTAATAATGGCG/ATAACGCGAAAAGCTTCCGC | 425 | 58 | *This work* |
| $bla_{ROB-1}$, $bla_{OXA-2}$ / $AMP^R$, $PEN^R$ | $bla_{ROB-1}$_F / $bla_{ROB-1}$_R | AATAACCCTTGCCCCAATTC/TCGCTTATCAGGTGTGCTTG | 685 | 60 | 18 |
| | $bla_{OXA-2}$ _F / $bla_{OXA-2}$ _R | GCAGACGAACGCCAAGCGGA/CCCGCACGATTGCCTCCCTC | 625 | 64 | *This work* |
| *erm*(42), *msr*(E), *mph*(E) / $TIL^R$, $TUL^R$ | *erm(*42)_F / *erm*(42)_R | GGGTGAAAAGGGCGTTTATT/ACGTTGCACTTGGTTTGACA | 1,254 | 60 | 50 |
| | *msr*(E)_F / *mph*(E)_R | ACCAGCCACCTTGATCTCAATG/GTTCCATTCGATCCAGTTATAGCG | 620 | 60 | *This work* |
| | *mph*(E)_F / *mph*(E)_R | TCTGTAGCGGGTTTCCAATTGC/AATGGTTGCTGCGTATTCCTCG | 401 | 60 | *This work* |
| *aadA25* / $SPT^R$ | *aadA25*_F / *aadA25*_R | GCAGTGGATGGCGGCCTGAA/TCGGCGCGATTTTGCCGGTT | 503 | 66 | *This work* |
| *aphA-1* / $NEO^R$ | *aphA-1*_F / *aphA-1*_R | TTATGCCTCTTCCGACCATC/GAGAAAACTCACCGAGGCAG | 489 | 54 | 51 |

| | | | | | |
|---|---|---|---|---|---|
| *strA*, *strB* / NEO$^{R}$,GEN$^{R}$ | *strA*_F / *strA*_R | AAGGCAAGGCGTTCGCGGTC/CCGGCGGCTGATCTGTCTGG | 506 | 64 | This work |
| | *strB*_F / *strB*_R | TCGCACCTGCTTGATCGCGG/GCTCGAATATGCCGGGGAGCG | 586 | 64 | This work |
| *aadB* / GEN$^{R}$ | *aadB*_F / *aadB*_R | TTACGCAGCAGGGCAGTCGC/ GCGGCACGCAAGACCTCAAC | 551 | 66 | This work |
| *floR* / FFN$^{R}$ | ICE *floR*_F / ICE *floR*_R | GACGGTTCGCGACGTTTATG/GAAGACGAAGAAGGTGCCCA | 320 | 58 | This work |
| *sul2* / TMP-SMX$^{R}$ | *sul2*_F / sul2_R | CCAATACCGCCAGCCCGTCG/TGCCTTGTCGCGTGGTGTGG | 489 | 64 | This work |
| ***Integrative conjugative element (ICE) associated genes from ICEPmu1*** | | | | | |
| hypothetical protein | Pmu_02680F/Pmu_02680R | TTATGAACCCGGTGCGAGTG/TGTGAGAGCAAAGACTCTGGT | 226 | 58 | This work |
| integrase | Pmu_02700F/Pmu_02700R | ACGGAATCATAGACCTGCCAC/TCTGTTGCAGTTGTATGTCGGA | 735 | 58 | This work |
| multicopper oxidase | Pmu_03360F/Pmu_03360R | CAAGGCAGTGCTGGGACATA/GTTCCTTGCGTTTCACCCAC | 458 | 58 | This work |
| transposase *tnpA* | Pmu_03510F/Pmu_03510R | TGCCGCTTTTCGTCTTTGTG/TACACGCCGAAGTTTCCGAA | 204 | 56 | This work |
| single stranded DNA-binding protein | Pmu_03540F/Pmu_03540R | GACTTCTCGACGTTCTCCGG/ATCGTTGCAATTTCCTGTCC | 110 | 58 | This work |

[a] F and R denote forward and reverse primers. P denotes probe

[b] RT denotes one-step Reverse Transcription-PCR primers

[c] N denotes second round Nested PCR primers

### 3.2.2 DNA and RNA extractions

Total DNA and RNA were extracted according to manufacturer's specifications from lung tissue samples using the QIAamp DNA tissue kit and RNeasy Mini Kit (Qiagen Canada Inc., Toronto, ON), respectively. Total nucleic acid (DNA extraction without RNase treatment) was extracted from nasopharyngeal swabs using the following procedures: nasopharyngeal swabs were centrifuged at 13,000 x g for 5 min and the supernatant was aspirated. The swab was removed from the applicator and left inside the tube containing the pellet, resuspended in 180 µL of enzymatic lysis buffer and incubated for 30 min at 37°C. A 25 µL aliquot of proteinase K and 200 µL of Buffer AL (provided with kit) were added to the suspension and incubated at 56°C for 30 min. A sterile steel ball was added to the tube and samples were processed for 1 min at 30 Hz using a Tissue Lyser (Qiagen Canada Inc., Toronto, ON). A 200 µL volume of ethanol (96-100%) was added and the suspension centrifuged at 8000 × g for 5 min to pellet debris. The supernatant was passed through a DNeasy spin column and nucleic acids were eluted as described in the kit protocol. All samples were stored at -80°C until used.

### 3.2.3 Viral and bacterial pathogen detection

All viral and bacterial agents were detected by PCR using master mixes according to manufacturer's specification and primers and annealing temperatures listed in Table 3.1. An overall profile of respiratory tract occurrence was generated with an animal identified as positive if the agent was detected in the nasopharynx, lung or both. Lung DNA and nasopharyngeal nucleic acid extractions were used as templates for PCR detection of *Mannheimia* sp., *P. multocida* and *H. somni*, nested PCR detection of BHV-1 and real-time PCR detection employing TaqMan® Fast Universal PCR Master Mix (Life Technologies Inc., Burlington, ON) of *M. bovis* (Sachse et al., 2010). Lung RNA and nasal nucleic acid extractions were used as templates for PCR detection of BVDV, BRSV, and $PI_3V$, employing SuperScript III One-Step RT-PCR System (Life Technologies Inc., Burlington, ON) for reverse transcription followed by a nested PCR. A HotStar HiFidelity Polymerase Kit (Qiagen Canada Inc., Toronto, ON) was used for nested PCR of BVDV reverse transcription products, with generated amplicons sequenced (Eurofins MWG Operon, Huntsville, AL) and analyzed using Geneious R6 version 6.0.5 (Biomatters Ltd., Auckland, New Zealand). Alignments generated in BLAST were used for identification of BVDV subspecies 1 and 2.

DNA extracted from the following strains was used for bacterial positive controls: *Pasteurella multocida ss multocida* strain 17976B (CCUG), *M. haemolytica* strain BAA-410 (ATCC), *Histophilus somni* strain 700025 (ATCC) and *Mycoplasma bovis* strain 25523 (ATCC). Positive controls for viral agents were prepared by cloning specific PCR products into plasmids using the first round PCR primers listed in Table 3.1. Amplicons generated from nucleic acids extracted from viral strains (BVDV1, Singer strain; BVDV2,

Ames 125-C strain; BHV-1, Colorado-34 strain) or positive nasopharyngeal/lung sample (BRSV and $PI_3V$) were cloned into a TA vector pCR™2.1 (Life Technologies Inc. Burlington, ON). Cloned fragments were confirmed by sequencing.

**3.2.4 Statistical analysis**

Pearson correlation analysis (SAS System for Windows, release 9.1.3, SAS Institute, Cary, NC) was used to determine the correlation between PCR results from nasopharyngeal and lung tissue samples for all bacterial and viral agents examined in this study and the level of significance was set at $P < 0.01$.

**3.2.5 Serotyping**

*Mannheimia haemolytica* isolates were serotyped using the rapid plate agglutination procedure with rabbit antisera raised against reference strains of *M. haemolytica* as previously described (Klima et al., 2011).

**3.2.6 Pulsed-field gel electrophoresis**

Molecular typing was performed on all isolates of *M. haemolytica*, *P. multocida* and *H. somni* by PFGE according to previous methods (Klima et al., 2011). Macrorestriction digests were completed using *Sal*I for *M. haemolytica*, *Apa*I for *P. multocida* and *Sac*II for *H. somni*. Electrophoresis conditions for all three species employed a program of 6 V/cm for 22 h at 12°C with switch times from 4-40 s. Pulsed-field profile and statistical analyses were performed using BioNumerics V5.1 software (Applied Maths, Inc., Austin, TX, USA).

**3.2.7 Broth microdilution assay**

Antimicrobial susceptibility testing was performed on *M. haemolytica*, *P. multocida* and *H. somni* isolates by broth microdilution using a commercially available panel (Bovine/Porcine with Tulathromycin MIC Format, Sensititre; Trek Diagnostic Systems, Cleveland, OH), as previously described for *M. haemolytica* (Klima et al., 2014) and according to manufacturer's specifications for *P. multocida* and *H. somni*. Minimum inhibitory concentration (MIC) was assigned by the unaided eye as outlined in the Clinical and Laboratory Standards Institute document M31-A3 (CLSI, 2008). Clinical and Laboratory Standard Institute breakpoints were not available for clindamycin, ampicillin, penicillin, gentamicin, neomycin, tiamulin, sulphadimethoxine, trimethoprim/sulfamethoxazole or tylosin tartrate. Consequently, susceptibility designations for these drugs were not assigned. Exceptions were made in cases where ampicillin, penicillin, neomycin or gentamicin MIC distributions for the population were bimodal and isolates exhibited both a high MIC and harbored a corresponding antimicrobial resistance gene determinant. In these instances, isolates were classified as resistant. In addition, the resistance

breakpoint for spectinomycin (≥128 µg/mL) was greater than evaluated by the panel (max. concentration evaluated was 64 µg/mL). As a result, isolates were classified as spectinomycin resistant if they exhibited both a high MIC and harbored a corresponding antimicrobial resistance gene determinant.

**3.2.8 Screening for resistance determinants and Integrative conjugation elements (ICE)**

PCR was used to screen for resistance gene determinants and ICE associated genes from ICE*Pmu1* in isolates of *M. haemolytica, P. multocida* and *H. somni.* All PCR reactions used heat lysed colony suspension as template and primers and annealing conditions described in Table 3.1. Representative PCR products were sequenced to confirm the accuracy of amplified fragments.

**3.2.9 Bacterial conjugation assay**

Conjugal mating experiments were performed to examine the mobility of ICEs among isolates of *M. haemolytica, P. multocida* and *H. somni*. Spontaneous rifampicin resistant ($Rif^R$) isolates for *P. multocida* strain 17976B and *Escherichia coli strain* K12 were generated by selecting and purifying colonies on BHI or Luria-Bertani (LB; BD Canada Inc., Mississauga, ON) agar plates, respectively, containing 25 µg/mL rifampicin. The $Rif^R$ *P. multocida* was used as a recipient for *M. haemolytica* and *H. somni* donor strains while $Rif^R$ *E. coli* was used as recipient for *P. multocida* donor strains. Overnight cultures of donor and recipient strains were grown at 37°C on TSA plates containing 5% blood and mating assays were performed as previously described (Michael et al., 2012b). Representative colonies of resulting transconjugants were streak-purified twice before the transfers of resistance conferring elements was verified by susceptibility testing and PCR screening for resistance determinants and ICE associated genes from ICE*Pmu1*. To ensure that transconjugants were not spontaneously resistant donor cells, they were characterized by PCR using the species-specific primers listed in Table 3.1.

**3.3 Results**

**3.3.1 Bacterial and viral pathogen detection**

For the detection of bacterial and viral pathogens implicated in BRD (Table 3.2; BVDV, BRSV, BHV-1, $PI_3V$, *Mannheimia* sp., *M. bovis*, *P. multocida* and *H. somni*) nucleic acid extractions from lung tissue and nasopharyngeal swabs were screened by PCR and used to generate an overall profile of their occurrence within the respiratory tract. Samples were identified as positive if the viral or bacterial agent was detected in the nasopharynx, lung or both. *Mannheimia* sp. were present in 91% of cattle, followed by BVDV (69%) with the subspecies BVDV1 accounting for 77% of BVDV positives. BVDV2 was observed in 26% of cattle, with all BVDV2 containing samples originating from Alberta. One animal screened positive for both BVDV subspecies, with BVDV2 detected in the nasopharynx and BVDV1 detected in the

lung. *Mycoplasma bovis* (63%) and *H. somni* (57%) were detected in more than half of the mortalities, followed by BRSV (19%), $PI_3V$ (13%) and *P. multocida* (13%). BHV-1 was not detected in any of the samples. Significant positive correlations were found between PCR results from nasopharyngeal swab and lung tissue samples in individual animals for *Mannheimia* sp. ($r = 0.391$, $p = 0.0011$), *P. multocida* ($r = 0.531$, $p<0.0001$), *M. bovis* ($r = 0.395$, $p = 0.0010$) and BVDV ($r = 0.630$, $p<0.0001$).

Table 3.2 PCR and culture based detection of bovine respiratory disease agents from BRD mortalities originating in Alberta, Nebraska and Texas[a]

| Species | Sample Origin[b] | | | Detected in both sample types (%) | Total (% Total) |
|---|---|---|---|---|---|
| | Alberta (% Alberta) | Nebraska (% Nebraska) | Texas (% Texas) | | |
| | | *PCR data* | | | |
| *Mannheimia haemolytica* | 38 (90) | 19 (95) | 5 (83) | 48 (77) | 62 (91) |
| *Pasteurella multocida* | 6 (14) | 1 (5) | 2 (33) | 3 (33) | 9 (13) |
| *Histophilus somni* | 25 (60) | 8 (40) | 6 (100) | 14 (35) | 39 (57) |
| *Mycoplasma bovis* | 23 (55) | 15 (75) | 5 (83) | 22 (51) | 43 (63) |
| Parainfluenza 3 virus | 6 (14) | 2 (10) | 1 (17) | 1 (11) | 9 (13) |
| Bovine respiratory syncytial virus | 10 (24) | 2 (10) | 1 (17) | 2 (15) | 13 (19) |
| Bovine herpes virus 1 | - | - | - | - | - |
| Bovine viral diarrhea virus (BVDV) | 29 (69) | 17 (85) | 1 (17) | 33 (70.2) | 47 (69) |
| BVDV-1 | 17 (41) | 17 (85) | 1 (17) | - | 35 (51) |
| BVDV-2 | 11 (27) | - | - | - | 11 (16) |
| BVDV-1/BVDV-2 | 1 (2) | - | - | - | 1 (1) |
| | | *Culture data* | | | |
| *Mannheimia haemolytica* | 31 (74) | 20 (100) | 4 (67) | - | 55 (81) |
| serotype 1 | 22 (52) | 18 (90) | 4 (67) | - | 44 (65) |
| serotype 2 | 1 (2) | 1 (5) | - | - | 2 (3) |
| serotype 6 | 8 (20) | 1 (5) | - | - | 9 (13) |
| *Pasteurella multocida* | 6 (14) | 1 (5) | 1 (16) | - | 8 (12) |
| *Histophilus somni* | 5 (12) | 2 (10) | 3 (50) | - | 10 (15) |

[a] Total PCR detection data and data broken down by sample origin are overall respiratory tract profiles, positive if agent was present in either nasopharyngeal swab sample, lung tissue sample or both. Detection in both sample types represent number of positive animals with PCR detected agent in both nasopharyngeal swab and lung tissue sample. Culture data represent isolates recovered from lung tissue samples

[b] Alberta $n$ = 42, Nebraska $n$ = 20, Texas $n$ = 6 , Total $n$ = 68

Co-existence of multiple pathogens was found in 97% of BRD cases with *Mannheimia* sp., BVDV and *M. bovis* co-occurrence most frequently identified (16.2%), followed by *Mannheimia* sp., BVDV, *M. bovis* and *H. somni* co-occurrence (11.8%) and *Mannheimia* sp. and BVDV co-occurrence (10.3%) (Figure 3.1). Overall, the most prevalent pathogens identified together were *Mannheimia* sp. and BVDV, being present in 63% of the cattle studied.

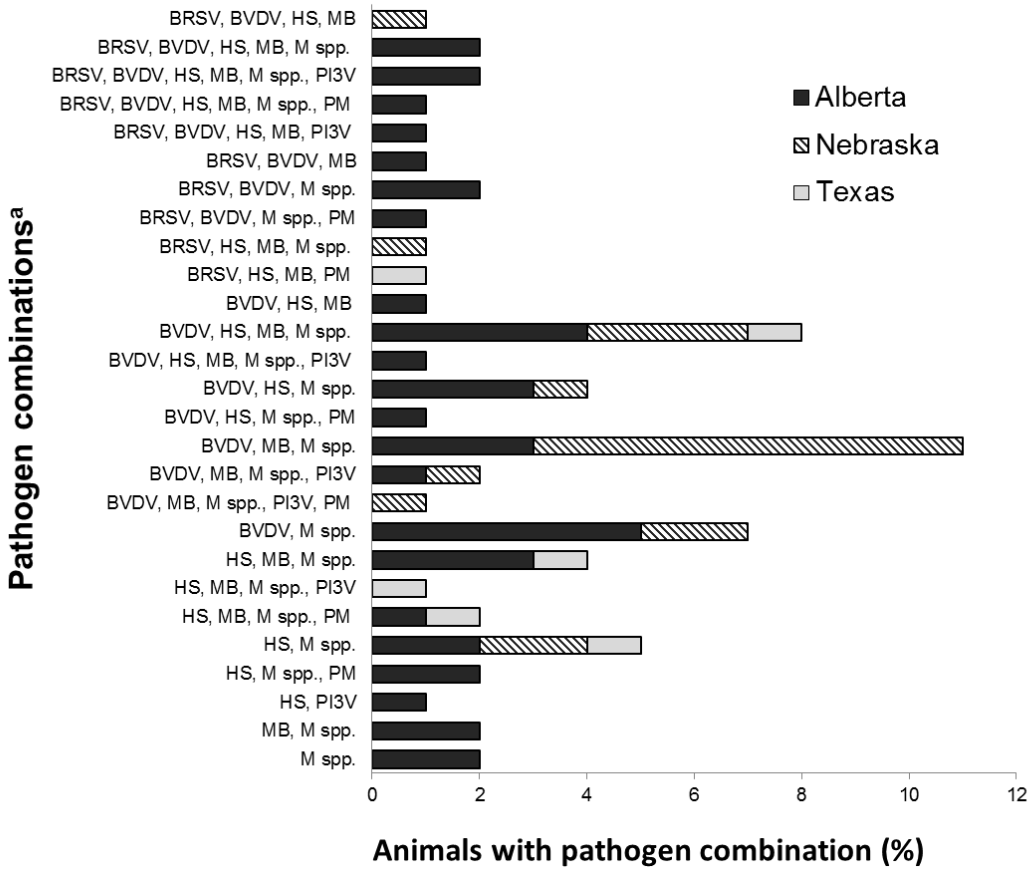
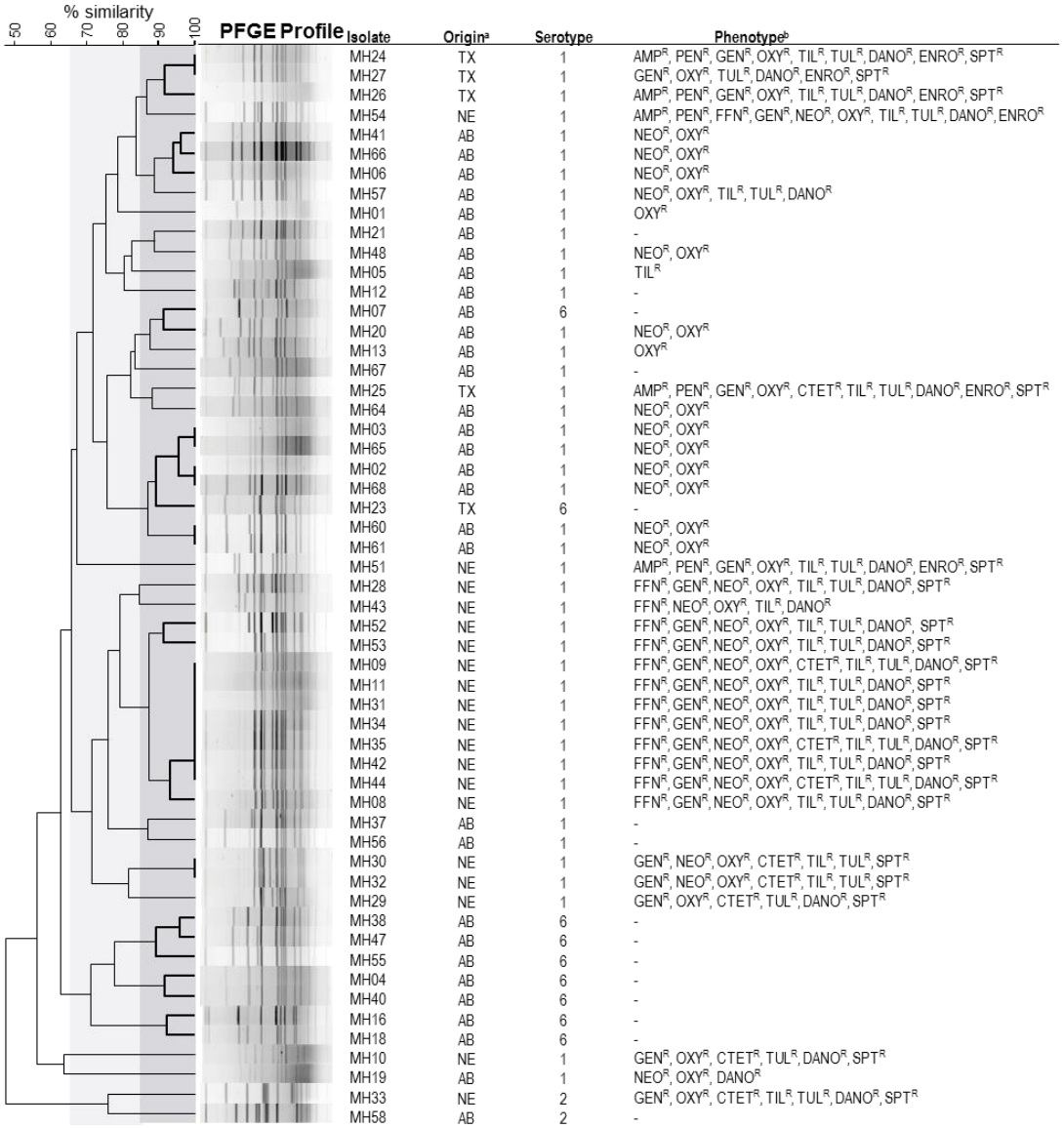



Figure 3.1 Bacterial and viral co-infection in the respiratory tract of feedlot cattle mortalities that succumbed to bovine respiratory disease. [a]BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhea virus; HS, *Histophilus somni;* MB, *Mycoplasma bovis;* MH, *Mannheimia haemolytica;* PM, *Pasteurella multocida;* PI3V, parainfluenza 3 virus. Overall respiratory tract profiles are presented with animals classified as infected if pathogenic agents were detected by PCR in nasopharyngeal swab sample, lung tissue sample or both.

Bacterial species that were identified by their 16S rRNA sequences, but not part of the BRD complex included *Bacillus* sp*., Psychrobacter* sp*., E. coli, Carnobacterium* sp*., Carnobacterium* sp*., Enterobacter* sp*., Trueperella pyogenes, Pantoea agglomerans, Comamonas sp., Aeromonas* sp*., Alcaligenes* sp*., Arthrobacter oxydans, Lactobacillus sakei, Pasteurella trehalosi, Proteus mirabilis, Staphylococcus vitulinus* and *Streptococcus uberis* (data not shown).

**3.3.2 Isolate recovery and characterization**

Single isolates of *M. haemolytica* (*n* = 55), *P. multocida* (*n* = 8) and *H. somni* (*n* = 10) were recovered from lung tissue samples (Table 3.2). From the 55 *M. haemolytica* isolates collected across North America, 80% (*n* = 44) were serotype 1, 3.6% (*n* = 2) were serotype 2 and 16.3% (*n* = 9) were serotype 6. PFGE analysis identified a clonal subpopulation of *M. haemolytica* (*n* = 8) in isolates originating in Nebraska (Figure 3.2; MH09, MH11, MH31, MH34, MH35, MH42, MH44, MH08). Antimicrobial resistant isolates of *P. multocida* were found in cattle from Texas, Nebraska and Alberta and shared > 70% genetic relatedness (Figure 3.3A). Isolates of *H. somni* from Alberta and Texas exhibited near identical pulsotypes (Figure 3.3B).




| Isolate | Origin[a] | Serotype | Phenotype[b] |
|---|---|---|---|
| MH24 | TX | 1 | AMP$^R$, PEN$^R$, GEN$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, ENRO$^R$, SPT$^R$ |
| MH27 | TX | 1 | GEN$^R$, OXY$^R$, TUL$^R$, DANO$^R$, ENRO$^R$, SPT$^R$ |
| MH26 | TX | 1 | AMP$^R$, PEN$^R$, GEN$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, ENRO$^R$, SPT$^R$ |
| MH54 | NE | 1 | AMP$^R$, PEN$^R$, FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, ENRO$^R$ |
| MH41 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH66 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH06 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH57 | AB | 1 | NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$ |
| MH01 | AB | 1 | OXY$^R$ |
| MH21 | AB | 1 | - |
| MH48 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH05 | AB | 1 | TIL$^R$ |
| MH12 | AB | 1 | - |
| MH07 | AB | 6 | - |
| MH20 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH13 | AB | 1 | OXY$^R$ |
| MH67 | AB | 1 | - |
| MH25 | TX | 1 | AMP$^R$, PEN$^R$, GEN$^R$, OXY$^R$, CTET$^R$, TIL$^R$, TUL$^R$, DANO$^R$, ENRO$^R$, SPT$^R$ |
| MH64 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH03 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH65 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH02 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH68 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH23 | TX | 6 | - |
| MH60 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH61 | AB | 1 | NEO$^R$, OXY$^R$ |
| MH51 | NE | 1 | AMP$^R$, PEN$^R$, GEN$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, ENRO$^R$, SPT$^R$ |
| MH28 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH43 | NE | 1 | FFN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, DANO$^R$ |
| MH52 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH53 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH09 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, CTET$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH11 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH31 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH34 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH35 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, CTET$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH42 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH44 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, CTET$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH08 | NE | 1 | FFN$^R$, GEN$^R$, NEO$^R$, OXY$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH37 | AB | 1 | - |
| MH56 | AB | 1 | - |
| MH30 | NE | 1 | GEN$^R$, NEO$^R$, OXY$^R$, CTET$^R$, TIL$^R$, TUL$^R$, SPT$^R$ |
| MH32 | NE | 1 | GEN$^R$, NEO$^R$, OXY$^R$, CTET$^R$, TIL$^R$, TUL$^R$, SPT$^R$ |
| MH29 | NE | 1 | GEN$^R$, OXY$^R$, CTET$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH38 | AB | 6 | - |
| MH47 | AB | 6 | - |
| MH55 | AB | 6 | - |
| MH04 | AB | 6 | - |
| MH40 | AB | 6 | - |
| MH16 | AB | 6 | - |
| MH18 | AB | 6 | - |
| MH10 | NE | 1 | GEN$^R$, OXY$^R$, CTET$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH19 | AB | 1 | NEO$^R$, OXY$^R$, DANO$^R$ |
| MH33 | NE | 2 | GEN$^R$, OXY$^R$, CTET$^R$, TIL$^R$, TUL$^R$, DANO$^R$, SPT$^R$ |
| MH58 | AB | 2 | - |

Figure 3.2 Pulsotype, serotype and antimicrobial susceptibility profile of *Mannheimia haemolytica* cultured from lung tissue of bovine respiratory disease mortalities in North America. Dendrogram created using UPGMA clustering of Dice coefficient values. Similarity matrix based on band-matching analysis, optimization and tolerance settings of 1.0% and 1.5%, respectively. [a] TX, Texas; NE, Nebraska; AB, Alberta. [b]AMP$^R$, ampicillin resistant; PEN$^R$, penicillin resistant; GEN$^R$, gentamicin resistant; OXY$^R$, oxytetracycline resistant; TIL$^R$, tilmicosin resistant; TUL$^R$, tulathromycin resistant; DANO$^R$, danofloxacin resistant; ENRO$^R$, enrofloxacin resistant; SPT$^R$, spectinomycin resistant; CTET$^R$, chlortetracycline resistant; FFN, florfenicol resistant.

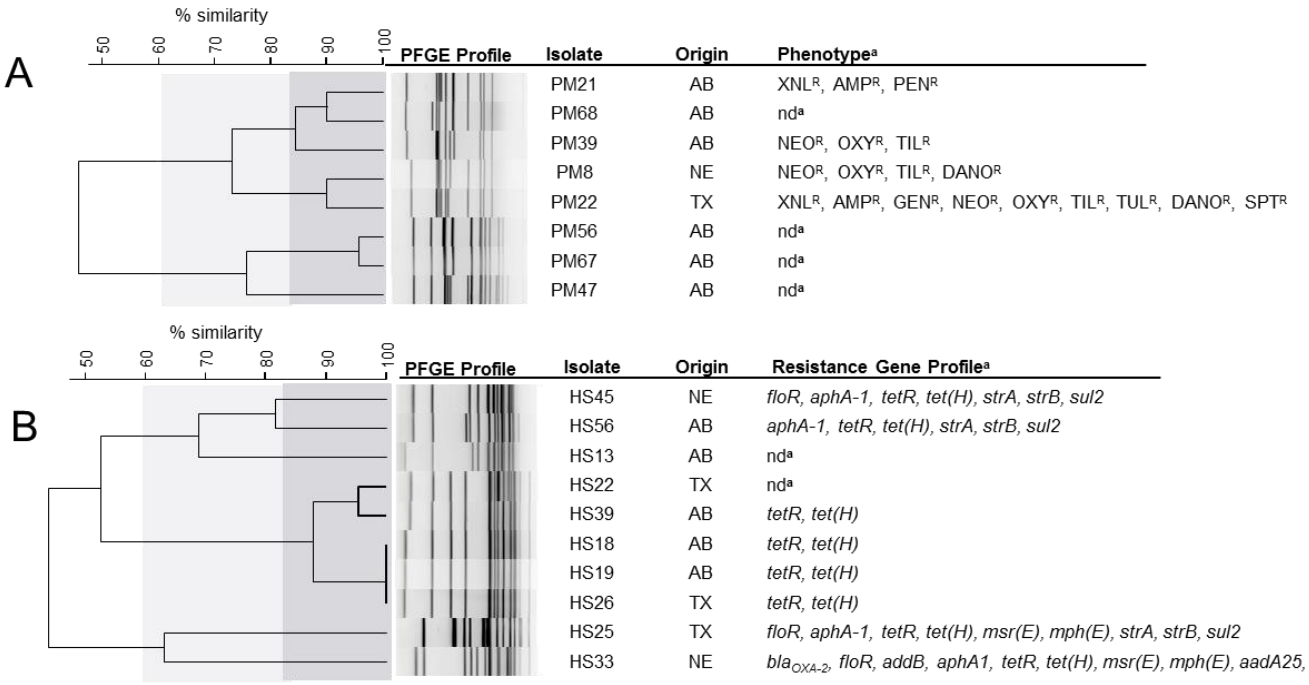



Figure 3.3 Pulsotype and antimicrobial susceptibility and resistance gene determinant profiles of (A) *Pasteurella multocida* and (B) *Histophilus somni* cultured from lung tissue samples from bovine respiratory disease mortalities in North America. Dendrogram created using UPGMA clustering of Dice coefficient values. Similarity matrix was based on band-matching analysis, optimization and tolerance settings of 1.0% and 1.5%, respectively. Phenotype data presented for *P. multocida* only. [a]nd none detected; $XNL^R$ ceftiofur resistant, $AMP^R$, ampicillin resistant; $PEN^R$, penicillin resistant; $GEN^R$, gentamicin resistant; $OXY^R$, oxytetracycline resistant; $TIL^R$, tilmicosin resistant; $TUL^R$, tulathromycin resistant; $DANO^R$, danofloxacin resistant; $SPT^R$, spectinomycin resistant. Resistance gene profile for *H .somni* generated from PCR detection of resistance determinants listed in Table 1.

Susceptibility testing showed 72% of *M. haemolytica* and 50% of *P. multocida* isolates were antimicrobial resistant. Of these, 30% of *M. haemolytica* (Figure 3.2; *n* = 16) and 12.5% of *P. multocida* (Figure 3.3A; *n* = 1) were resistance to more than seven antimicrobial classes including aminoglycosides, penicillins, fluoroquinolones, lincosamides, macrolides, pleuromutilins and tetracyclines. All multidrug resistant isolates originated from samples collected in Texas or Nebraska. Antimicrobial resistant phenotypes were observed in multiple *M. haemolytica* populations with pulsotypes ranging in similarity from 65-100% (Figure 3.2), indicating that resistance was not spread strictly by clonal dissemination. All antimicrobial resistance phenotypes observed corresponded with PCR-positive results for the ICEs associated genes from ICE*Pmu1* listed in Table 3.3 with the exception of two *P. multocida* isolates for which a determinant for ceftiofur resistance was not identified.

Table 3.3 Donor strain gene profile, transfer outcome and resulting transconjugant genotype and phenotype

| Donor Strain | Donor strain resistance gene profile | Donor strain ICE associated genes from ICE*Pmu1* profile[a] | Transconjugant matches donor yes[b,c]/ no[d] |
|---|---|---|---|
| MH2 | *aphA-1, tetR, tet(H) , strA, strB, sul2* | Cu-oxi, tnpA, ssb | no[a] |
| MH8 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, *tnpA, ssb* | no[a] |
| MH9 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH10 | *$bla_{OXA-2,}$ aadB, tetR, tet(H), msr(E), mph(E), aadA25* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH11 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH24 | *$bla_{ROB-1,}$ $bla_{OXA-2,}$ aadB, tetR, tet(H), msr(E), mph(E), aadA25* | Cu-oxi, tnpA, ssb | yes[b] |
| MH25 | *$bla_{ROB-1,}$ $bla_{OXA-2,}$ aadB, tetR, tet(H), msr(E), mph(E), aadA25* | Cu-oxi, tnpA, ssb | no[a] |
| MH26 | *$bla_{ROB-1,}$ $bla_{OXA-2,}$ aadB, tetR, tet(H), msr(E), mph(E), aadA25* | Cu-oxi, tnpA, ssb | no[a] |
| MH27 | *$bla_{OXA-2,}$ addB, tetR, tet(H), msr(E), mph(E), aadA25* | Cu-oxi, tnpA, ssb | no[a] |
| MH28 | *$bla_{OXA-2}$, floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH29 | *$bla_{OXA-2,}$ addB, tetR, tet(H), msr(E), mph(E), aadA25* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH30 | *$bla_{OXA-2,}$ addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH31 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH32 | *$bla_{OXA-2,}$ addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH33 | *$bla_{OXA-2,}$ addB, tetR, tet(H), msr(E), mph(E), aadA25* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH34 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB,* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH35 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH42 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH43 | *floR, aphA-1, tetR, tet(H), erm(42), strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH44 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH51 | *$bla_{ROB-1,}$ $bla_{OXA-2,}$ aadB, tetR, tet(H), msr(E), mph(E), aadA25,* | Cu-oxi, tnpA, ssb | no[a] |
| MH52 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH53 | *$bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| MH54 | *$bla_{ROB-1,}$ $bla_{OXA-2,}$ floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Cu-oxi, tnpA, ssb | yes[b] |
| MH57 | *aphA-1, tetR, tet(H), strA, strB, sul2* | Cu-oxi, tnpA, ssb | no[a] |
| PM8A | *aphA-1, tetR, tet(H), erm(42), strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| PM22A | *$bla_{OXA-2}$, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), addA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes[c] |

| | | | |
|---|---|---|---|
| PM39A | *aphA-1, tetR, tet(H), erm(42), strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| HS25A | *floR, aphA-1, tetR, tet(H), msr(E), mph(E), strA, strB, sul2* | Hypo, Int, tnpA, ssb | no[a] |
| HS33A | *$bla_{OXA-2}$, floR, addB, aphA-1, tetR, tet(H), erm(42), msr(E), mph(E), aadA25, strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| HS45A | *floR, aphA-1, tetR, tet(H), strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |
| HS56A | *aphA-1, tetR, tet(H), strA, strB, sul2* | Hypo, Int, Cu-oxi, tnpA, ssb | yes |

[a]Hypo, hypothetical protein; Int, integrase; Cu_oxi, multicopper oxidase; tnpA, transposase tnpA; ssb, single stranded DNA-binding protein
[b]element did not transfer; [c]*$bla_{ROB-1}$* not detected; [d]ceftiofur resistant phenotype not observed

Antimicrobial susceptibility testing of *H. somni* isolates produced inconsistent results and therefore, only genotypic analyses were reported (Figure 3.3B). Polymerase chain reaction for resistance determinants revealed four isolates harboring multiple resistance genes (HS45, HS56, HS25, HS33; Figure 3.3B) and one isolate exhibiting a gene profile similar to the multidrug resistant isolates of *M. haemolytica and P. multocida* described above (HS33; Figure 3.3B).

**3.3.3 Conjugal transfer of ICE/mobile genetic elements**

Mating assays were used to assess the transfer of mobile genetic elements conferring multidrug resistance from isolates of *M. haemolytica* (*n* = 25) and *H. somni* (*n* = 4) to a $Rif^R$ strain of *P. multocida* and from *P. multocida* (*n* = 3) to a $Rif^R$ strain of *E. coli* (Table 3.3). All but eight matings successfully generated transconjugant colonies. Susceptibility testing and PCR were used to confirm phenotypes and genetic transfer of resistance determinants and ICE associated genes from ICE*Pmu1* to transconjugants. Danofloxacin resistance in donor stains was not observed to be transferred to recipients, possibly because this determinant was not located on the ICE or was not functional in transconjugants. In 21 of 24 successful transformations, all resistance phenotypes and associated resistance genes were transferred, excepting danofloxacin. In instances where successfully transformed recipients differed from the profiles of the donors, two transconjugants acquired all genes and corresponding resistance phenotype with the exception of the $bla_{ROB-1}$ gene and associated ampicillin and penicillin resistance (MH24, MH5; Table 3.3). One transconjugant acquired all resistance phenotypes with the exception of ceftiofur which was observed in the donor *P. multocida* strain (PM22; Table 3.3). In the eight cases where matings were unsuccessful, one donor isolate (MH8; Table 3.3) had the full complement of ICE associated genes from *ICEPmu1,* six of the donor isolates (Table 3.3; MH2, MH25, MH26, MH27, MH51, MH57) did not contain the hypothetical protein or integrase genes and one (HS25; Table 3.3) did not contain the multicopper oxidase gene as screened for by PCR. However, two isolates with a partial complement of ICE associated genes from ICE*Pmu1* (containing three of the five screened genes: multicopper oxidase, transposase *tnpA*, single stranded DNA-binding protein) were successfully transferred (MH54, MH24; Table 3.3).

**3.4 Discussion**

With the exception of a few cases (Booker et al., 2008; Fulton et al., 2009; Fulton et al., 2000), epidemiological data associated with acute BRD mortalities seldom include the complete range of agents that can play a role in the disease. Further, the prevalence of agents is often highly variable within individual cases, likely due to the multiple factors involved in BRD pathogenesis. Consistently, in all studies including ours, *M. haemolytica* has been the predominant pathogenic agent detected, and is

often identified in mixed-infections with *M. bovis* and BVDV (Booker et al., 2008; Fulton et al., 2009; Fulton et al., 2000). The occurrence of BVDV in this study was slightly higher than expected. Administration of modified live vaccines is a potential source of false positive data if the duration between vaccination and testing is too short and the vaccine virus is still detectible by PCR. Although the majority of the cattle in this study received a modified live vaccine, more than 90% were sampled at least seven days after the vaccine was administered upon entry to the feedlot. Persistently infected (PI) cattle are also known to be a primary source of BVDV exposure, leading to seroconversion in over 80% of previously virus free calves post contact (Ridpath et al., 2000). Although not screened for in the feedlots sampled, PI animals could have contributed to the increased occurrence of BVDV seen here. It was also interesting to note that all cases of BVDV2 were found in Alberta when BVDV2 has been detected in 27.9% of US stocker calves with acute respiratory disease (Fulton et al., 2000). Others have also shown that BVDV2 accounts for a substantial portion of the North American BVDV population (Ellis, 2009). It is possible the failure to detect BVDV2 in USA BRD mortalities reflects a lower prevalence rate of BVDV2 compared to Canada. It is also possible that data are a result of the restricted sample size, a limitation of the PCR to identify cattle infected with multiple strains of BVDV or the result of the use of BVDV2 based modified live vaccines in Alberta, but not in USA Feedlots.

Compared to previous investigations (Fulton et al., 2009; Welsh et al., 2004), the occurrence of *P. multocida* was lower than anticipated. However, it is acknowledged that *P. multocida* plays a significant role in dairy calf pneumonia and in subacute/chronic bronchopneumonia (Dabo et al., 2007; Panciera and Confer, 2010) and less of a role in acute fibrinous pneumonia in feedlot calves. Despite selection for fibrinous pneumonia cases, *M. bovis* and *H. somni* were isolated at higher rates than expected; comparable to those detected in chronic and bronchiolar pneumonia cases in Alberta (Booker et al., 2008). Likewise, the prevalence of BSRV in the current study was more similar to that reported in subacute pneumonia (Booker et al., 2008). In the past five years, chronic pneumonia has accounted for an increasing proportion of reported BRD cases (Woolums, 2010) and it is not uncommon to see both fibrinous and caseonecrotic pneumonia occurring simultaneously within the same individual (Gagea et al., 2006). Additionally, co-isolation of multiple respiratory pathogens is commonly reported in BRD diagnostic investigations (Booker et al., 2008; Fulton et al., 2009; Fulton et al., 2000; Lamm et al., 2012). Thus, the elevated detection of some pathogens not typically associated with fibrinous pneumonia may not be surprising.

The only viral pathogen tested for, but not detected in this study was BHV-1. Previously, BHV-1 has been reported in up to 10% of BRD cases (Booker et al., 2008; Fulton et al., 2009). However, if

calves had been infected with BHV-1 prior to sampling, the virus may not have been detected as it persists latently in the sensory neurons of the trigeminal ganglia or tonsils (Ellis, 2009) and is undetectable in nasal mucus post infection (Pastoret and Thiry, 1985). Our limited sample size may also have contributed to our low level of detection of this pathogen.

Previously, ribotyping of *P. multocida* obtained from nasal and tracheal swabs of calves with BRD found that 70% of the isolates obtained from these two locations were genetically identical (DeRosa et al., 2000) and Timsit et al. (2013) recently found that 77% of *M. haemolytica* collected from the nasopharynx and trans-tracheal aspirations had identical PFGE profiles. Here, we were interested to see if nasopharyngeal swabs could serve as a reliable and economical alternative to lung tissue to detect both viral and bacterial agents implicated in BRD using PCR. Comparisons of PFGE profiles or DNA sequences from *M. haemolytica* and BVDV respectively, collected from both the nasopharynx and lungs of individual animals, revealed that in over 80% of cases the agents were identical (data not shown). Further, high concordance was observed between nasopharyngeal swabs and lung tissue for PCR detection of *Mannheimia* sp. and BVDV, indicating that nasopharyngeal swabs may provide a representative profile of the involvement of these agents in acute BRD mortalities.

Between 2000 and 2009, overall decreases in the susceptibility of *M. haemolytica* to danofloxacin, tilmicosin, tulathromycin, enrofloxacin and florfenicol have been observed, with the latter two antimicrobials in particular associated with increased resistance in *H. somni* (Portis et al., 2012). Over time, the MICs of tetracycline, tilmicosin and tulathromycin have also been increasing for *H. somni* (Portis et al., 2012), whereas bovine sourced *P. multocida* has shown reduced susceptibility to florfenicol, spectinomycin, tetracycline, tilmicosin and trimethoprim/sulphamethoxazole (Welsh et al., 2004). Consistent with these trends, the present study revealed a high overall rate of antimicrobial resistance. Seventy-two percent of *M. haemolytica* isolated in this study were antimicrobial resistant, about twice that detected for *M. haemolytica* isolated from the nasopharynx of cattle displaying BRD (35%) in a previous study from our group (Klima et al., 2014). Furthermore, evidence for multidrug resistance against more than three antimicrobials was found in 45% of the isolates collected during this project; agreeing with reports that multidrug resistance in BRD pathogens is on the increase (Esaki et al., 2004; Lubbers and Hanzlicek, 2013; Tang et al., 2009).

Recently, an isolate of *P. multocida* originating from a beef calf in Nebraska was shown to harbour an ICE (ICE*Pmu1*) that carried 11 resistance determinants (Michael et al., 2012a). The similarity between the antimicrobial resistant phenotypic profile of ICEP*mu1* and the isolates recovered in this study prompted the search and subsequent detection of ICE in *M. haemolytica, P. multocida* and *H.*

*somni.* Integrated conjugative elements are a diverse group of mobile genetic elements found in both Gram-positive and Gram-negative bacteria, including Proteobacteria, Actinobacteria and Firmicutes. Although resident in the host chromosome, ICEs retain the machinery required for excision and conjugal transfer (Wozniak and Waldor, 2010). These elements contribute to the horizontal transfer of accessory genes that can bestow traits of antibiotic and heavy metal resistance, virulence, biofilm formation, nitrogen fixation, and metabolic adaptation (Bi et al., 2012; Wozniak and Waldor, 2010). The modular structure of ICEs with tandem arrays of closely related elements promotes generation of novel ICEs through module swapping and recombination (Wozniak and Waldor, 2010). Certain ICE families employ processes such as toxin-antitoxin systems to prevent their loss, ensuring reintegration back into the donor cell after conjugation has occurred (Wozniak and Waldor, 2009). Integrated conjugative elements have been detected in *H. somni* with accessory genes for heavy metal and toxin resistance (Michael et al., 2012b), but prior to this report they have not contained multidrug resistance cassettes. In *M. haemolytica,* a partial ICE was identified by Michael et al. (2012b) in the draft genome sequence of PHL213, and a putative ICE*Mh*1 was recently detected in *M. haemolytica* strain 42548; the latter harbouring five resistance genes*, aphA-1*, *strA*, *strB, sul2* and *tetR-tetH* (Eidam et al., 2013), a less developed complement of resistance genes than that identified in the present study.

ICE*Pmu1* from *P. multocida* has been shown to undergo conjugal transfer among strains of *M. haemolytica* and *E. coli* (Michael et al., 2012b). In this study, we successfully transferred ICEs from *M. haemolytica* and *H. somni* into a recipient strain of *P. multocida,* but not directly into *E.coli*. Transformation efficiency was not examined here and it is possible that ICE transfer from *M. haemolytica* to *E. coli* or from *H. somni* to *E. coli* occurs, but at low frequency. Integrated conjugative elements from *P. multocida* were readily transferred into a recipient strain of *E. coli,* raising the possibility that *P. multocida* may act as a potential vector for ICEs among bacterial species.

Further investigations into interspecies transfer of the ICEs detected here, in particular transfer from BRD pathogens into enteric zoonotic or environmentally persistent species that thrive in fecal material and water bodies in feedlot environments, are important. Transfer of ICEs into environmental isolates, such as those we co-isolated with BRD pathogens (*Actinobacter* sp., *Bacillus* sp., *Carnobacterium* sp. and *Psychrobacter* sp.), could result in the dissemination of these elements into the broader environment, establishing a reservoir for their persistence. In addition, many veterinary and zoonotic pathogens are present in the feedlot environment including *Salmonella* sp., *Enterococci* sp., *Campylobacter jejuni, E. coli*, *Yersinia enterocolitica* and *Clostridium difficile*. Potentially pathogenic species including *E. coli*, *Proteus mirabilis*, *Moraxella bovoculi* and *Streptococcus uberis* were identified

in this study, but not examined for antimicrobial resistance phenotypes. Multidrug resistance development in these organisms as a result of a single ICE transfer event could potentially have undesirable consequences for human health.

In this study it was interesting that multidrug resistant ICEs were detected in a high proportion of the USA samples (88%) but not from the samples screened from Alberta. In the USA, the majority of feedlots contain more than 32,000 animals, with the five largest feeding operations controlling approximately 20% of feeding capacity. The average feedlot in Alberta or Saskatchewan contains approximately 8000 head with 64% of feedlots in Alberta having capacities of less than 20,000 head (Galyean et al., 2011). It is possible that larger, higher density operations require more antimicrobial input to prevent disease. Likewise, the USA calves sampled were on average 250 lbs lighter and on feed 17 days less than those in Alberta, indicating that they were likely considered high risk for developing BRD and targeted for metaphylactic treatment upon arrival.

It is important to note that BRD mortalities were targeted in this study and the cattle examined were likely exposed to more antimicrobial therapies than the general cattle population. Further, sample size was a limitation and a larger surveillance project would be required to comment on the prevalence of ICE-harbouring bacteria in North American cattle populations. Future investigations into antimicrobial resistance profiles of *P. multocida*, *M. haemolytica* and *H. somni* from swine and poultry operations and at other levels of the beef production system, including cow-calf and various points during the marketing chain would be advisable. Aarestrup et al. (2008) estimated that swine and poultry account for about 34% and 33%, respectively, of the global livestock antimicrobial market, whereas cattle account for only 26% (Aarestrup et al., 2008). The majority of ICEs in this study could be co-selected for through the use of florfenicol, enrofloxacin, tilmicosin, or tetracycline; all drugs used to prevent or treat BRD (Welsh et al., 2004), but they are also approved for use in swine and poultry. Examination of the prevalence of ICE-harbouring bacteria in the general feedlot population would be of benefit as rates of resistance have been shown to differ between healthy and sick populations of cattle (Klima et al., 2014) and the high levels of resistance observed in mortality cases here may not be represent those of the general population.

To our knowledge, this is the first detection of ICEs in *M. haemolytica* and *H. somni* from USA feedlots and the very recent identification of ICE*Pmu1* (Michael et al., 2012a) indicates that these elements may have recently emerged within the feedlot environment. In the current study, isolates of *M. haemolytica* were obtained that exhibited resistant phenotypes against all antimicrobials commonly employed to treat BRD, except ceftiofur. However, resistance to ceftiofur was observed in this study in

an isolate of *P. multocida* and the potential incorporation of this determinant into existing *M. haemolytica* could result in pathogenic isolates resistant to the entire suite of therapies commonly used to treat and prevent respiratory disease in feedlot cattle.

**3.5 Conclusion**

The identification of ICEs in this study is new information that may affect our thought processes relative to how antimicrobials are used for BRD control and treatment. One hypothesis is that the presence or absence of ICEs relates to antimicrobial use, however in the current study the Alberta feedlots followed the same antimicrobial use protocols for BRD control and treatment as those in Nebraska. As such, continued research should be supported to investigate interspecies barriers for ICE transfer and to describe donor vs. recipient range to provide more information about how features of these elements contribute to proliferation and spread. Further, comparative sequence analysis of these identified ICE is required to confirm gene arrangement, and may give insight into of the evolutionary development of these specific elements. If ICEs were to become established in bacterial populations implicated in BRD, it could compromise the ability of prophylactic use of antimicrobials to control this important disease.

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# 4 Chapter 4: Draft genome sequence of a *Mannheimia haemolytica* serotype 6 isolate collected from the nasopharynx of a beef calf with bovine respiratory disease

Chapter 4 has been published in Genome announcements.

Klima, C.L., Cook, S.R., Hahn, K.R., Amoako, K.K., Alexander, T.W., Hendrick, S., McAllister, T.A., 2013. Draft Genome Sequence of a *Mannheimia haemolytica* Serotype 6 Isolate Collected from the Nasopharynx of a Beef Calf with Bovine Respiratory Disease. Genome announcements 1.

This manuscript was drafted by Cassidy Klima with suggestions and comments from the collaborating authors. Experimental design, data collection and analysis were undertaken by Cassidy Klima and Dr. Tim McAllister.

**4.1 Introduction and results**

*Mannheimia haemolytica,* a member of the family *Pasteurellaceae,* is a commensal in the upper respiratory track of birds and mammals and can contribute to acute fibrinous pleuropneumonia. In feeder cattle, this is termed bovine respiratory disease (BRD) and costs the North American industry millions yearly through treatment, reduced meat yield and mortalities.

Host specificity and virulence of *M. haemolytica* is linked to serovar, with serotypes 1 (S1) and 6 (S6) being infectious to cattle (Rice et al., 2007). Whole genomes of serotypes 1 (Gioia et al., 2006) and 2 (S2) (Lawrence et al., 2010), have been sequenced, but not S6. We describe a draft genome sequence of *M. haemolytica* H23, a S6 isolate obtained from the nasopharynx of a beef calf with BRD.

The *M. haemolytica* H23 genome was sequenced by Cofactor Genomics (St Louis, MO) using both Roche 454 Jr. and Illumina Genome Analyzer IIx. A total of 99,677 single-end 400bp 454 reads and 47,372,771 paired-end 80bp Illumina reads were generated, with respective coverages of 14x and 2847x. Independent assemblies using Newbler 2.5p1 (454) and SOAPdenovo (Illumina) were merged with Minimus2. A draft genome of 69 contigs with a total of 2,662,064 bp, a GC content of 40.8% and $N_{50}$ of 101,714 bp was produced and verified using optical mapping.

Gene prediction and function using the Integrated Microbial Genomes (IMG; http://img.jgi.doe.gov/) platform (Markowitz et al., 2010) identified 2704 genes representing 89.2% of total bp. Of these, 2628 were protein coding sequences, 2197 with assigned functions; 416 for transporters, 764 signal peptides and 551 transmembrane proteins. Compared to S1 and S2 genomes, 52 genes coding for primarily hypothetical or phage-related proteins were exclusive to *M. haemolytica* H23. A CRISPR element and a type III secretory component identical to S2 (Lawrence et al., 2010) were also detected. Gene prediction identified 3 5S rRNA, 1 16S rRNA and 1 23S rRNA gene.

This Whole Genome was deposited at DDBJ/EMBL/GenBank under the accession AOGP00000000. The version described in this document is the first version, AOGP01000000.

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# 5 Chapter 5: Comparative genomic analysis of *Mannheimia haemolytica* from bovine sources

Chapter 5 is intended for submission

This manuscript was drafted by Cassidy Klima with suggestions and comments by Dr. Tim McAllister, Dr. Rahat Zaheer, and Shaun Cook. Experimental design, data collection and analysis were undertaken by Cassidy Klima and Dr. Tim McAllister.

**5.1 Introduction**

*Mannheimia haemolytica* is an important bacterial pathogen of ruminants and the principal agent of bovine respiratory disease (BRD) in feedlot cattle, a condition responsible for an annual loss of over 3 billion US$ to beef producers worldwide (Watts and Sweeney, 2010). An opportunistic pathogen, *M. haemolytica's* role in disease is strongly associated with serovar. Of the 12 capsular serotypes identified to date, serotype 1 (S1) and serotype 6 (S6) are the most prevalent in BRD (Al-Ghamdi et al., 2000; Rice et al., 2007). Serotype 2 (S2) is found frequently as a commensal in the upper respiratory tract of healthy cattle (Klima et al., 2011), but can produce pneumonia in ovine hosts, frequently causing high mortality rates that have devastated wild and domestic sheep populations (Lawrence et al., 2010b).

Multiple virulence factors have been characterized in *M. haemolytica*, the most significant being a secreted leukotoxin and a capsular lipopolysaccharide (Zecchinon et al., 2005). However, little is understood about how genetic differences among the serovars contribute to pathogenesis. Until recently, whole genome sequences were only available for one bovine strain of S1 and two strains of S2, one being from cattle, the other from a sheep (Gioia et al., 2006; Lawrence et al., 2010a). Although S1 is the principal serovar associated with BRD in North America, the prevalence of S6 in this disease has been increasing (Katsuda et al., 2008).

Treatment of BRD in cattle relies heavily on antibiotics, making *M. haemolytica* a primary target for these therapies in feedlots. As a result, there is risk for selection of antimicrobial resistant *M. haemolytica* within this setting. The recent isolation of pan-resistant *M. haemolytica* isolates from beef calves (Eidam et al., 2015; Lubbers and Hanzlicek, 2013) is of concern both for maintaining drug efficacy and the risks associated with resistance gene transfer between bacterial species in the agriculture sector. Such a risk is particularly relevant to zoonotic pathogens that can impact human health. Consequently, there is a need to develop less drug dependant strategies to control BRD.

Veterinary vaccines play a major role in the management of disease and in conferring protection against pathogens, however the development of effective vaccines against BRD has been challenging. Comparative analysis between pathogenic and commensal serovars of *M. haemolytica* allows for the examination of virulence mechanisms that have not been phenotypically defined and can aid in identifying targets for vaccine design. To better understand the genetic factors contributing to virulence we sequenced and performed comparative genomic analysis on 11 strains of *M. haemolytica* representing serotypes 1, 2 and 6 from cattle that were healthy, morbid or deceased as a result of BRD.

## 5.2 Materials and methods

### 5.2.1 Isolate selection

Eleven isolates of *M. haemolytica* representing serotypes 1, 2 or 6, from healthy, pneumonic or cattle that succumbed to BRD were subject to whole genome sequencing (Table 5.1). These were selected from an archive of 532 isolates collected between 2007 and 2012 that had been previously characterized with pulsed-filed gel electrophoresis and antimicrobial susceptibility testing (Klima et al., 2011; Timsit et al., 2013, as described in Chapters 2 and 3). Candidates for sequencing were selected based on criteria that maximized diversity in both genotypes and antimicrobial resistance profiles, but also considered strains collected from a variety of geographical locations including Canada (Alberta), USA (Nebraska, Texas) and France (Nantes). Isolates collected from lung tissue, nasopharyngeal swabs or tracheal aspirations were all represented (Table 5.1).

Table 5.1 Features of 11 *Mannheimia haemolytica* strains sequenced

| Strain | Serovar | Sample type | Animal status | Sampling Location | Genomes Size Mb | No. Contigs | CDS | No. prophage (% of genome) | No. CRISPR Spacers | ICE size kb (%GC) |
|---|---|---|---|---|---|---|---|---|---|---|
| *M. haemolytica* L024A | 1 | lung | deceased | Texas | 2.64 | 116 | 2768 | 8 (11.3) | 14 | 70.6 (40.2) |
| *M. haemolytica* L044A | 1 | lung | deceased | Nebraska | 2.6 | 122 | 2714 | 5 (6.9) | 15 | 81.1 (41.6) |
| *M. haemolytica* 157-4-1 | 1 | nasopharynx | healthy | Alberta | 2.6 | 116 | 2715 | 7 (11.6) | 14 | 48.6 (39.3) |
| *M. haemolytica* 535A | 1 | nasopharynx | morbid | Alberta | 2.58 | 158 | 2730 | 6 (10.5) | 13 | 47.1 (39.3) |
| *M. haemolytica* T2 | 2 | lung | morbid | France | 2.43 | 107 | 2448 | 2 (4.1) | 8 | n/a |
| *M. haemolytica* L033A | 2 | lung | deceased | Nebraska | 2.57 | 128 | 2639 | 4 (8.2) | 7 | 66.3 (40.2) |
| *M. haemolytica* 587A | 2 | nasopharynx | healthy | Alberta | 2.5 | 111 | 2547 | 5 (8.3) | 4 | n/a |
| *M. haemolytica* L038A | 6 | lung | deceased | Alberta | 2.6 | 134 | 2724 | 6 (10.9) | 16 | 50.4 (39.1) |
| *M. haemolytica* T14 | 6 | trachea | morbid | France | 2.56 | 101 | 2647 | 5 (9.2) | 17 | 49.9 (39.1) |
| *M. haemolytica* H23 | 6 | nasopharynx | morbid | Alberta | 2.6 | 69 | 2628 | 4 (7.6) | 14 | 48.6 (39.3) |
| *M. haemolytica* 3927A | 6 | nasopharynx | healthy | Alberta | 2.52 | 105 | 2602 | 5 (7.4) | 16 | 46.6 (39.4) |

### 5.2.2 DNA extraction and sequencing

Phenol: chloroform extraction was used to isolate genomic DNA. Briefly, *M. haemolytica* cells from a 5 mL culture grown overnight in trypticase soya broth were harvested by centrifugation at 6000 x g for 5 min. The pellet was suspended in 1 mL of sterile 0.85 % NaCl and centrifuged again for 1 min to wash away growth media. The washed cell pellet was resuspended in 700 µL of $T_{10}E_{25}$ (10mM Tris-HCl pH7.5; 25mM EDTA). To the suspension, 175 µL of 5 M NaCl, 35 µL of 10 mg/mL Proteinase K and 44 µL of 20% SDS was added and the mixture was incubated at 65°C for 1-2 h until cells were completely lysed. The lysed mixture was extracted once with phenol, once with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform. Ammonium acetate (10 M) was added to the final aqueous fraction to achieve a final concentration of 0.5 M followed by the addition of 1 volume of isopropanol to precipitate the DNA. The DNA was gently spooled out, added to a new tube containing ice-chilled 70% ethanol and centrifuged at 10,000 x g for 10 min to obtain a DNA pellet. The supernatant was decanted and the tube was left open to air-dry. The pellet was suspended in 100 µL of nuclease free deionized water.

Genomic library construction and sequencing of the serotype 6 strain *M. haemolytica* H23 (Table 5.1) was performed by Cofactor Genomics (St Louis, MO). A draft genome was generated from combined assemblies of Roche 454 Jr. single-reads and paired-end reads generated using Illumina Genome Analyzer IIx (as discussed in chapter 4). This strategy was undertaken to generate a robust assembly and maximize sequence coverage in order to produce as high quality a draft genome as possible as this was the first S6 *M. haemolytica* strain sequenced. Library construction and sequencing of the remaining 10 *M. haemolytica* isolates was performed using Roche-454 GS FLX Titanium chemistry by Genome Québec (McGill University, QC) with draft genomes assembled using Newbler software. With the exception of *M. haemolytica* H23, which was annotated through the NCBI Prokaryotic Genome Annotation Pipeline (PGAAP) (Angiuoli et al., 2008), gene prediction and functional annotation of all other genomes were performed using the Integrated Microbial Genomes (IMG) platform (Markowitz et al., 2009).

### 5.2.3 Comparative analysis

Sequences of the 11 draft genomes were aligned using Mugsy (Angiuoli and Salzberg, 2011). The core, accessory and pan-genomes were calculated using the pan-genomes analysis pipeline (PGAP) (Zhao et al., 2012). Cluster analysis cut-off values were set at 85% sequence identity over 90% of the sequence length. To examine the pan-genome of *M. haemolytica* with an expanded dataset, the analysis was also performed including an additional 10 *M. haemolytica* strains from public databases

(Table A1 in Appendix A). Single nucleotide polymorphism (SNP) discovery and validation was performed using in-house scripts on filtered *454AllDiffs.txt* files, using the serovar 1 *M. haemolytica* USDA-ARS-USMARC 183 strain as the reference. A SNP atlas was produced using Circos (Krzywinski et al., 2009) depicting the single point differences compared to the reference genome *M. haemolytica* USDA-ARS-USMARC 183 (Figure B1 in Appendix B). For bacteriophage analysis, contigs were ordered based on alignment against *M. haemolytica* strain M42548 using progressive Mauve (Darling et al., 2010), with the resulting concatenated contigs analyzed for the presence of prophage using PHAST (Zhou et al., 2011). Prophages were aligned using MAFFT (Katoh and Standley, 2013) and phylogenetic analysis generated using neighbor tree reconstruction in Phylip v. 3.68 (Sanchez et al., 2011). Genes not annotated by PHAST were examined using PSI BLAST (Altschul et al., 1997) and IMG (Markowitz et al., 2013). Proposed arrangements for integrative conjugative elements (ICEs) were constructed by pairwise alignments against ICE*Pmu*1 and ICE*Mh*1 (GeneBank accession numbers: CP003022, CP005383, respectively). In-house scripts were used to BLAST unassembled reads against previously assembled ICEs where possible to verify gaps. CRISPR-Cas regions were compiled using the CRISPRdb (Grissa et al., 2007). Alignments of individual prophage, CRISPR-Cas, ICEs and virulence genes were performed using Geneious version 6.1.6. Where provided, reference locus IDs originated from *M. haemolytica* H23 (AOGP01000042). This genome was used as a reference as it has already been published and submitted to NCBI. For cases when a locus ID was not available from *M. haemolytica* H23, reference locus IDs were supplied from the first fully closed genome of *M .haemolytica,* strain M42548 (CP005383).

**5.3 Results and discussion**

**5.3.1 Sequencing statistics**

Sequencing of the 11 *M. haemolytica* strains resulted in draft genomes ranging from 2.43Mb to 2.60Mb, with assemblies containing 69-158 contigs (Table 5.1). Compared to S2 strains, S1 and S6 genomes contained on average, 100kb more genomic content, which was mainly attributable to added or enlarged integrated mobile genetic elements such as integrative conjugative elements and prophage. Percent identity among the genomes ranges from 79.0% to 97.8% (Figure 5.1). Sequence diversity was highest amongst the three S2 genomes, ranging from 87.0% to 96.2% identity.

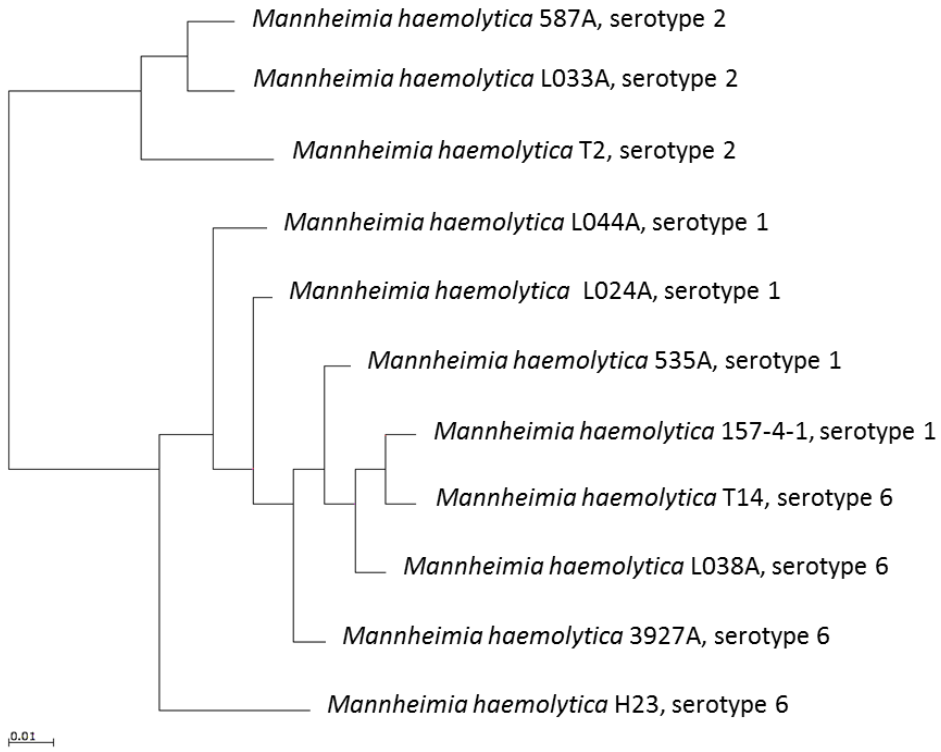


Figure 5.1 Dendrogram of 11 *Mannheimia haemolytica* genomes. Tree generated by hierarchical clustering based on coding sequences within genomes.

**5.3.2 Core, dispensable and pan-genome analysis**

Pan-genome analysis serves two main functions, the first being to define clusters of orthologue groups within a dataset. The purpose of this is to identify the pan-genome which is defined as the global compliment of genes that code for all of the proteins a species can produce (Tettelin et al., 2005). From this analysis, the core genome defined as the set of genes common to all strains, can also be determined, along with the dispensable genome that contains the genes present in two or more strains. The accessory genome can also be described which contains those genes present in the dispensable genome along with all strain specific genes unique to each isolate. Analysis of the different genome subsets can provide important insights into the evolution of a species and identify potentially important novel genes (Callister et al., 2008). The core genome is expected to be comprised largely of coding sequences (CDSs) that are essential for cell function and survival, most of which are derived from a common ancestor (Callister et al., 2008). The dispensable genome contains the bulk of the diversity in a species (Medini et al., 2005) and is more likely to contain genes obtained through horizontal gene transfer.

The second function of pan-genomic analysis is to determine the pan-genome status. First described by Tettelin et al. (2005) an open pan-genome is one where the addition of new strains to the dataset will continue to result in the accumulation of new genes. Conversely a closed pan-genome occurs when the addition of new strains eventually results in the number of strain specific genes converging at zero, this being the point where the dataset encompasses the number of genome sequences required to sufficiently characterize the species (Medini et al., 2005). Open pan-genomes are frequently observed in species that inhabit diverse ecological niches, have complex lifestyles and are prone to horizontal gene transfer (De Maayer et al., 2014; Tettelin et al., 2005), while closed pan-genomes exist in species from isolated, narrow niches or where recent divergence has led to limited genetic diversity within the gene pool (Tettelin et al., 2005). A key example of a closed pan-genome occurs with *Bacillus anthracis* where the sequencing of four strains was found to completely characterize the species (Medini et al., 2005). Defining the status of the pan-genome can be used to determine the inclusiveness of a genomic dataset. Estimating the genomic diversity within a population of isolates in this fashion can allow for prediction of the number of whole genome sequences required to fully characterize a clade of organisms (Tettelin et al., 2005; Vernikos et al., 2015).

Analysis of the core genome of the 11 *M. haemolytica* strains identified 1,594 genes, accounting for approximately 60% of each genome (Figure 5.2; Figure 5.3). The accessory genome contained almost 3x the number CDSs (4587) of the core genome and although the dispensable genome contained some genes associated with survival and maintenance, over three quarters were hypothetical genes of unknown function. Approximately 300 unique genes (ranging from 257 to 367) were present in each of the 11 strains; (Figure 5.2). These were mostly of a hypothetical nature or unidentified CDSs within prophage regions. Characterization of these unknown genes would provide additional insight into the function and biology of *M. haemolytica.*

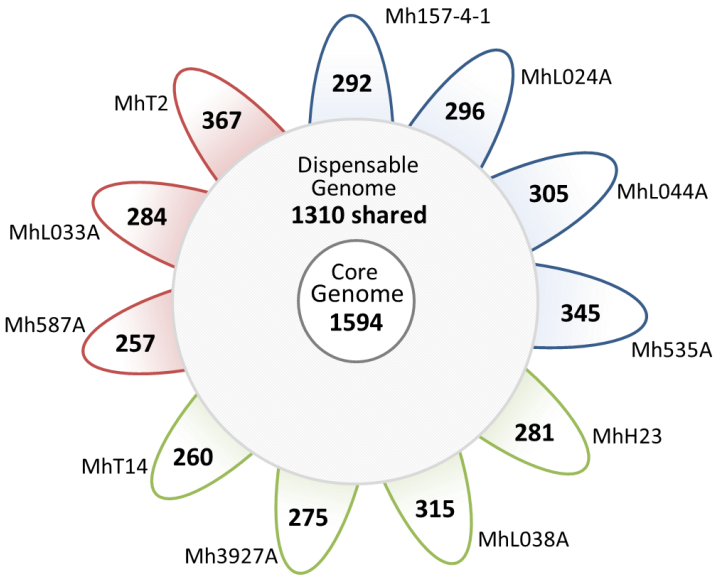


Figure 5.2 Size of the core genome, dispensable genome and number of strain unique CDS in 11 *Mannheimia haemolytica* genomes. Petals contain number of unique CDS per strain. Pink petals represent S2 strain, blue S1 strains and green S6 strains. Analysis based on 85% sequence identity across 90% length.

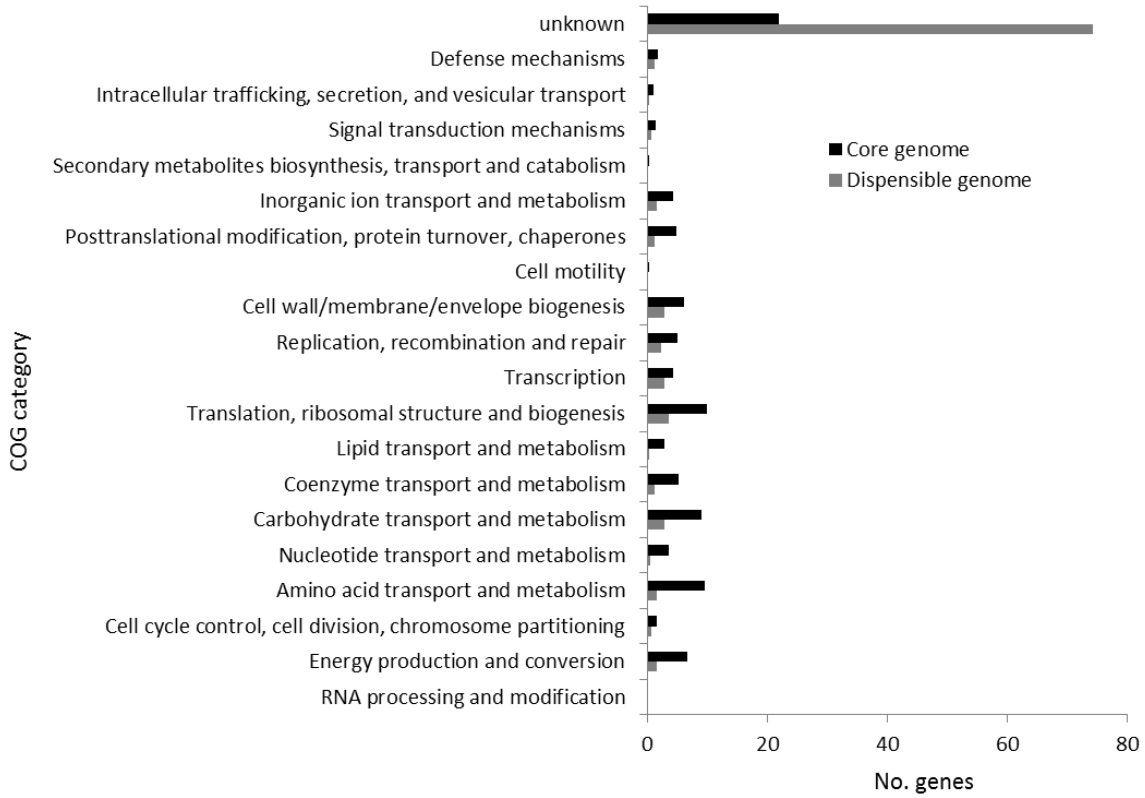


Figure 5.3 Percentage of genes from the core and dispensable genomes categorized based on Clusters of Orthologous Groups.

To obtain a more comprehensive perspective of the core- and pan-genomes of *M. haemolytica*, 10 publically available *M. haemolytica* genomes were included in the analysis along with the 11 isolates sequenced in the current work. This analysis determined the core genome of 21 *M. haemolytica* strains to contain 1,333 genes with the pan-genome remaining open, continuing to increase by approximately 286 new genes with the incorporation of each new strain (Figure 5.4 and Figure 5.5). Extrapolation of the curve fitted to the new gene plot for the 21 *M. haemolytica* strains estimated that even after the addition of 1000 genomes to the dataset, 123 unique genes per genome would still be added to the pan-genome. The pan-genome analysis when applied based on serovar showed that both S1 (n=8) and S6 (n=7) pan-genomes remained open (Figure 5.6A and Figure 5.6B), increasing with the addition of each new strain by 305 and 295 genes, respectively. However, the analysis shows the S2 pan-genome (n=6) to be closed with no new genes being added after the inclusion of six genomes (Figure 5.6C).

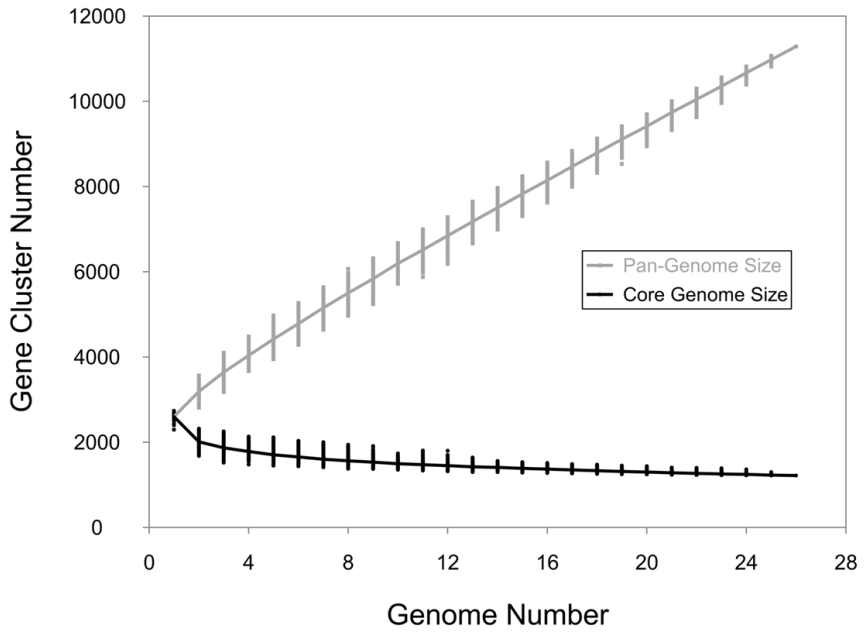


Figure 5.4 Pan and core genome of 21 *Mannheimia haemolytica* isolates. For the pan-genome the number of specific genes is plotted as a function of the number of strains sequentially added. For the core genome the number of shared genes is plotted as a function of the number of strains sequentially added.

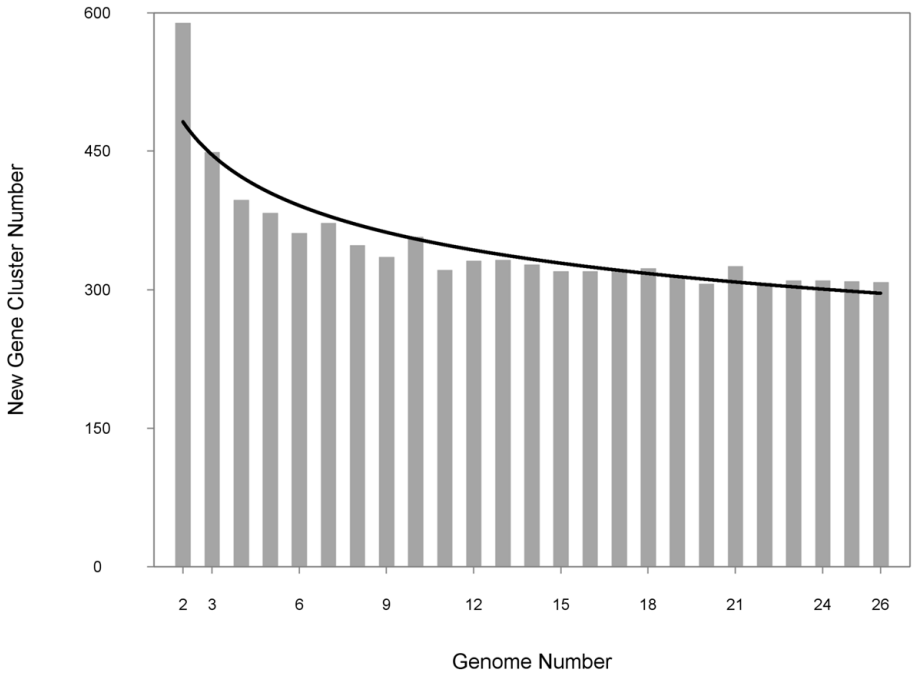

Figure 5.5 New gene discovery plot for pan-genome analysis of 21 *Mannheimia haemolytica* genomes

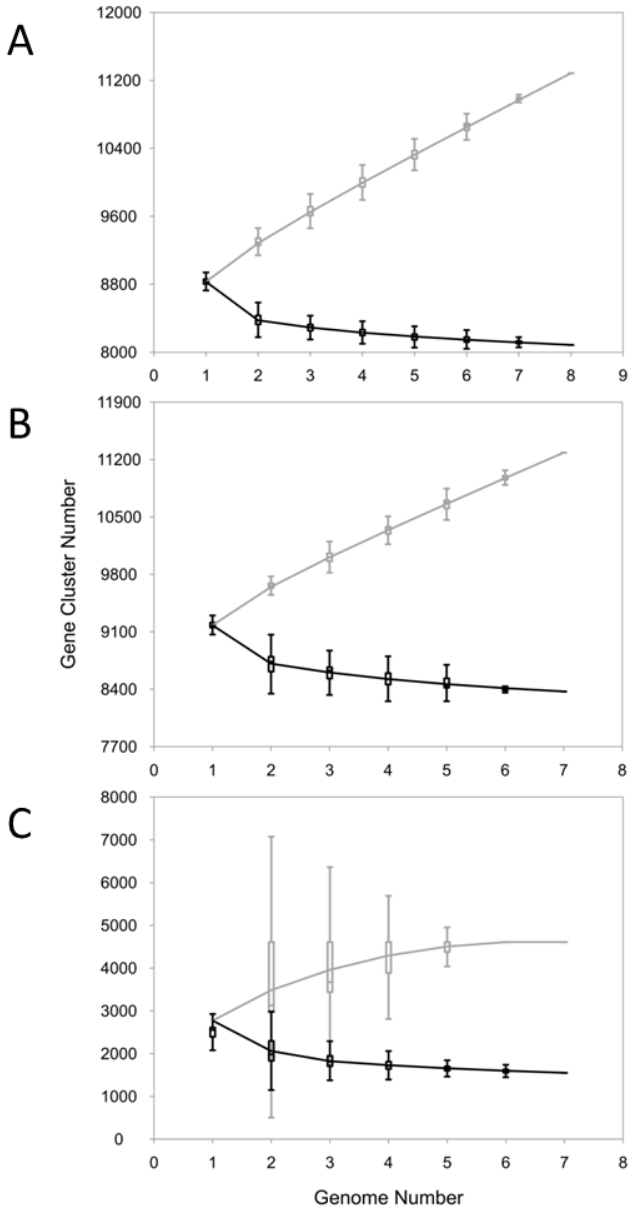


Figure 5.6 Pan and core genomes of *Mannheimia haemolytica* isolates based on serovar. For the pan-genome, in grey, the number of specific genes is plotted as a function of the number of strains sequentially added. For the core genome, in black, the number of shared genes is plotted as a function of the number of strains sequentially added. Panel A: serotype 1 strains, n= 8. Panel B: serotype 6 strains, n=7. Panel C: serotype 2 strains, n=6.

It was expected that the *M. haemolytica* pan-genome would be open given this bacterium inhabits a complex niche, can exhibit both commensal and pathogenic phenotypes, and contains many serovars with varying degrees of mobile genetic elements (MGE) (Gioia et al., 2006; Lawrence et al., 2010a). It was surprising that the S2 pan-genome was closed, especially when one considers that the

bulk of genomic diversity seen at the whole genome level occurs across S2 strains. It is clear the higher abundance of MGE within S1 and S6 strains contributes significantly to the diversity of unique CDSs in these serovars. The evolutionary basis for the overall lack of diversity and abundance of MGE in S2 compared with the pathogenic serovars remains unknown. It would be of interest to investigate if a closed pan-genome also occurs in S2 strains obtained from ovine sources. It is possible that a host species specific accessory gene pool occurs within the different ruminants and that potentially pathogenic MGE are more abundant in S2 strains associated with ovines.

**5.3.3 SNP analysis**

A total of 22,506 SNPs were identified and of these 20,833 were from annotated genes, with the remainder arising from intergenic regions (Table 5.2). The percentage of SNPs in each genome ranged from 0.0031 to 0.5722%, 31× and 16× higher in S2 than in S6 and S1 genomes, respectively. A total of 5,230 nonsynonymous SNPs (nsSNPs) were discovered in 1543 coding sequences, 2990 resulting in non-conservative amino acid substitutions (e.g. exchange of polar amino acid for a nonpolar amino acid) at 1208 loci. Over 80% of the nsSNPs occur within the S2 genomes with *M. haemolytica* T2 exhibiting the highest degree of divergence (Figure B1 in Appendix B). This is not unexpected as S2 strains have been shown to be highly diverse (Klima et al., 2011). Over 60% of nsSNPs occured within genes assigned to one of 6 COG categories including: unknown function (378), general function prediction only (147), carbohydrate transport and metabolism (101), amino acid transport and metabolism (97), replication, recombination and repair (97) and cell wall/membrane/envelope biogenesis (96) categories. The latter category is not surprising given greater diversity has been observed in outer membrane proteins among serotype 2 strains than either serotype 1 or serotype 6 strains (Davies and Donachie, 1996). Bacteriophage associated elements comprised a large proportion of the genes of unknown function.

Table 5.2 Single nucleotide polymorphism analysis of 11 *Mannheimia haemolytica* genomes using *M. haemolytica* USDA-ARS-USMARC 183 as reference

| | *M. haemolytica* genome | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | L044A | L024A | 157-4-1 | 535A | T14 | 3927A | L038A | H23 | L033A | 587A | T2 |
| Number of SNPs | 1804 | 1452 | 173 | 81 | 339 | 487 | 443 | 529 | 14724 | 13284 | 12195 |
| No. of sSNPs in coding regions | 1312 | 1076 | 68 | 29 | 150 | 181 | 152 | 156 | 10545 | 9401 | 8709 |
| No. of nsSNPs in coding region | 296 | 252 | 87 | 44 | 160 | 275 | 260 | 332 | 3100 | 2939 | 2729 |
| CDS with nsSNPs | 99 | 96 | 52 | 40 | 60 | 76 | 72 | 74 | 1183 | 1163 | 1034 |
| CDS with nsSNPS unique to strain | 60 | 46 | 19 | 17 | 42 | 23 | 28 | 38 | 311 | 292 | 634 |

A total of 72 nsSNPs that would result in premature stop codons were identified. These occurred most frequently in single genomes and over 50% were associated with genes of hypothetical function or phage regions (38). An outer membrane siderophore receptor (F388_05956) which is functional in S2 strains was predicted to be non-functional in three of the S1 strains. An additional six genes associated with iron acquisition (F388_09151, MHH_c20980, F388_03340, MHH_c28330, F388_10280, F388_03345) contain premature terminators making them non-functional in at least one of the S2 genomes. Given the importance of iron acquisition for pathogenesis and host adaptation in *M. haemolytica* (Roehrig et al., 2007) it is possible the mutations in these genes within the S2 genomes could play a role in reduced virulence of this serotype. The functional counterparts of these proteins in S1 and S6 strains would be interesting candidate antigens for a vaccine as they may be important for pathogenesis.

**5.3.4 Virulence factors**

*Mannheimia haemolytica* has multiple virulence factors that have been extensively studied and reviewed previously (Highlander, 2001). These include multiple fimbriae, adhesions, outer membrane proteins (Confer, 2009), neuraminidases (Griffin, 2010), transferrin-binding proteins and capsular polysaccharides (Rice et al., 2007). One of the most important elements of *M. haemolytica* pathogenesis is a secreted leukotoxin of the RTX repeat family that has cytolytic activity against bovine leukocytes (Highlander et al., 2000). Leukotoxin is produced by a locus containing four genes: *lktC, lktA, lktB,* and *lktD* (Davies et al., 2002; Rice et al., 2007). The *lktA* gene encodes for the inactive protoxin (pro-Lkt), the *lktC* gene encodes for a transacylase that post-translationally activates pro-LktA and the *lktB and lktD* genes code for a proteins that transport the active LktA from the cell (Davies et al., 2002; Rice et al., 2007; Thumbikat et al., 2005).

Seventy-two different genes with known roles in virulence were compared, the vast majority exhibiting >95% sequence identity among all sequences in the 11 *M. haemolytica* genomes (Table A2 in Appendix A). Genes with sequence identities <95% included TbpA and TbpB, which are part of a transferrin-iron uptake system, both the leukotoxin genes *lktA* and *lktC*, a surface antigen serotype-specific antigen 1 (Ssa1), the heme sequestering hemophore HuxA, the S-ribosylhomocysteinase LuxS which is involved in quorum sensing, the outer membrane protein PlpE, and WecC which is associated with LPS synthesis. Sequence variation between serovars for TbpA and TbpB (Lee and Davies, 2011), LktA and LktC (Davies and Baillie, 2003; Davies et al., 2002; Davies et al., 2001) and PlpE (Confer et al., 2006) have been documented previously. In all of these cases, sequence diversity within virulence genes occurs largely within the S2 strains, with genes in S1 and S6 strains nearly identical.

### 5.3.5 Mobile genetic elements

Horizontal gene transfer through transduction, transformation and conjugation is the primary means for prokaryotic organisms to obtain new genetic material (Wozniak and Waldor, 2010). Mobile genetic elements including plasmids, phages, genomic islands or genomic modules can transfer DNA that may alter the pathogenicity of an organism through the provision of toxins and antimicrobial resistance genes (Wozniak and Waldor, 2010). They may also provide factors that regulate virulence gene expression, influence adhesion or enhance immune evasion (Wagner and Waldor, 2002). As a result horizontal gene transfer plays an important role in the evolution, maintenance and transmission of virulence genes and the development and spread of antimicrobial resistance (Keen, 2012). Two types of MGE, ICE and prophage, were identified in the *M. haemolytica* genomes here, each contained genes with the ability to contribute to the virulence of the strains examined.

### 5.3.6 Bacteriophage

Prophage can be a significant source of genetic diversity in bacterial genomes and act as vectors for virulence factors including extracellular toxins, proteins involved in adhesion, modulation of host activity, resistance to serum and altered antigenicity, as well as mitogenic factors (Busby et al., 2013; Fortier and Sekulovic, 2013). Phage content in the genomes ranges from 4.1-11%, with a total of 57 intact prophages identified across the 11 sequenced strains, all clustering into 14 profiles based on nucleotide similarity (Figure 5.7; Table 5.3). Overall, S2 genomes contained fewer intact prophages (2-5) than S1 (5-8) or S6 (4-6) genomes. Clusters P1, P2, P3, P4, P7, P8, P13 and P14 resembled phage from the Siphoviridae family and clusters P6, P9, P10, P11, and P12 resembled phage from the Myoviridae family. Prophages vB_Mha-L024AP10 and vB_Mha-157-4-1P6 clustered independently contained fewer base pairs and although they contained multiple phage components they did not align against the reference strain *M. haemolytica* strain M42548, and thus may not represent true prophages.

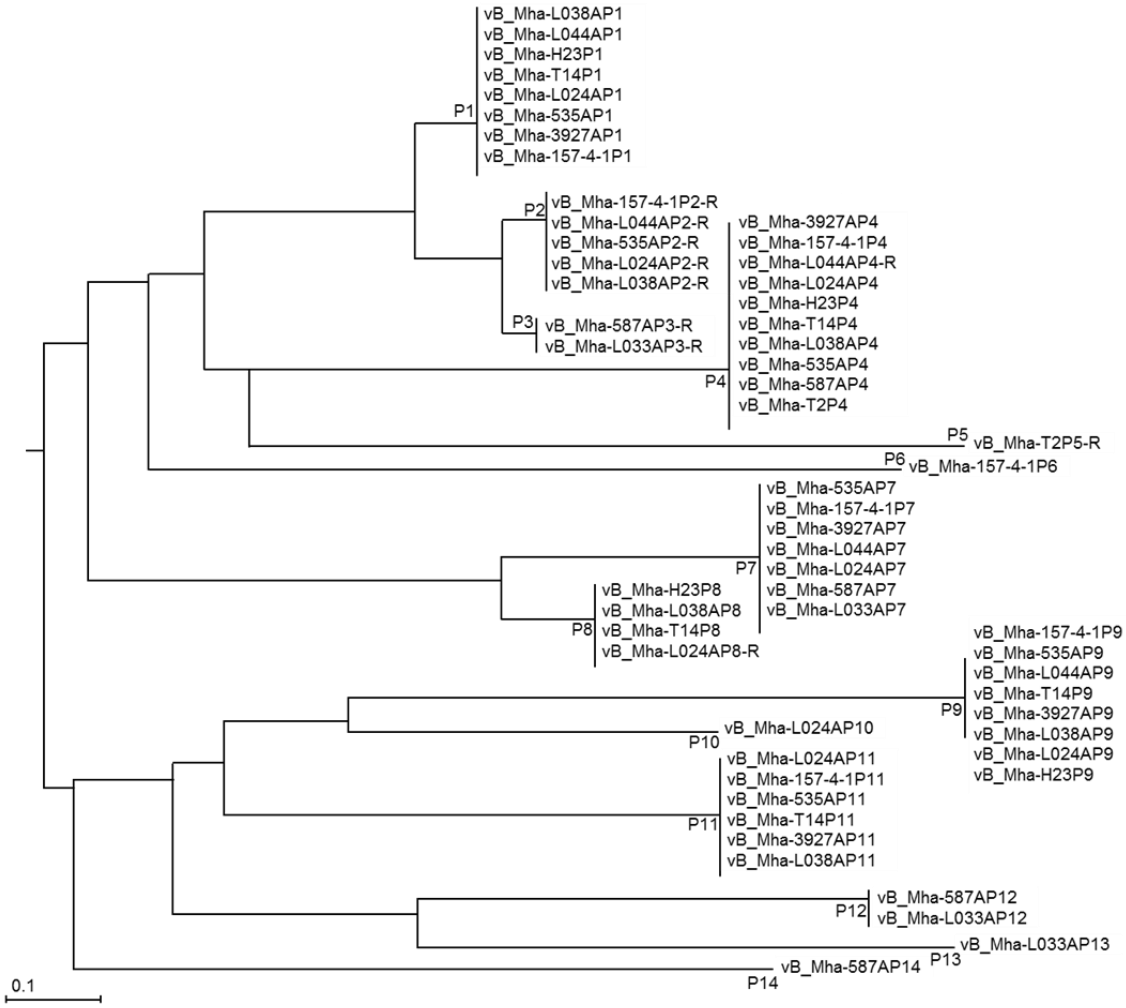


Figure 5.7 Phylogenetic analysis of 57 prophage found in 11 whole genome sequences of *Mannheimia haemolytica*. Phage cluster number identified at each node.

Table 5.3 Metadata for prophage found in *Mannheimia haemolytica*

| | | | | | | Prophage length in kb (No. CDS): | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cluster | Max length (kb) | Percent similarity | Spacer[a] | Toxin-antitoxin | Prophage family | 587A | L033A | T2 | L038A | H23 | T14 | 3927A | 154-7-1 | L044A | L024A | 535A |
| P1 | 42.7 | 95.3 | - | RelB/E | Siphoviridae | - | - | - | 38.5 (36) | 41 (39) | 38.9 (38) | 39 (38) | 30.9 (36) | 31.5 (36) | 39.9 (39) | 34.7 (35) |
| P2 | 56.1 | 92.2 | 31 | - | Siphoviridae | - | - | - | 51.1 (64) | - | - | - | 42.9 (62) | 42.1 (61) | 38.7 (59) | 53.9 (64) |
| P3 | 43.3 | 84 | 8, 28, 29, 30, 31 | HigA/B | Siphoviridae | 52.1 (81) | 104.1 (148) | - | - | - | - | - | - | - | - | - |
| P4 | 97.9 | 60.7 | 11, 12, 15 | - | Siphoviridae | 38.8 (45) | | 44.6 (52) | 76.1 (82) | 77.5 (83) | 76.9 (82) | 27.6 (29) | 76.1 (81) | 65.2 (82) | 76.2 (82) | 75.1 (84) |
| P5 | 54 | - | - | HigA/B | Siphoviridae | - | - | 53.9 (62) | - | - | - | - | - | - | - | - |
| P6 | 20.5 | - | - | HipA/ B | Myoviridae | - | - | - | - | - | - | - | 20.4 (26) | - | - | - |
| P7 | 54 | 67.1 | 3, 6, 17, 25 | HicB/C | Siphoviridae | 30.3 (51) | 35.4 (48) | - | - | - | - | 37.2 (52) | 48.8 (52) | 25.5 (43) | 27.6 (48) | 31 (46) |
| P8 | 39.1 | 59.7 | 3, 6, 16, 25 | HigA | Siphoviridae | - | - | - | 32.5 (51) | 37.4 (51) | 34.4 (46) | | - | - | 22.3 (24) | - |
| P9 | 47.1 | 94.5 | - | - | Myoviridae | - | - | - | 46.3 (60) | 41.8 (51) | 41.9 (57) | 42 (57) | 46 (60) | 46.2 (61) | 45.2 (61) | 40.9 (56) |
| P10 | 14.1 | - | - | HipB | Myoviridae | - | - | - | - | - | - | - | - | - | 14 (21) | - |
| P11 | 47.5 | 85.1 | 26, 27 | HipB | Myoviridae | - | - | - | 38.3 (51) | - | 43.4 (57) | 40.1 (54) | 34.8 (48) | - | 35 (49) | 35 (49) |
| P12 | 43.3 | 84 | - | - | Myoviridae | 41.9 (58) | 41.1 (56) | - | - | - | - | - | - | - | - | - |
| P13 | 29.3 | - | - | HicB/C | Siphoviridae | - | 29.3 (39) | - | - | - | - | - | - | - | - | - |
| P14 | 44.7 | - | - | HigA | Siphoviridae | 44.7 (47) | - | - | - | - | - | - | - | - | - | - |

[a]CRISPR spacer found with >90% sequence identity to phage sequence

Complete (P1, P3, P5, P6, P7, P13) or partial (P8, P10, P11, P14) toxin-antitoxin systems occurred in 10 of the 14 phage clusters identified. Toxin anti-toxin systems are small genetic modules that consist of an operon coding for a stable toxin that inhibits cell growth and an unstable antitoxin that protects against the toxin's effects (Van Melderen and De Bast, 2009; Wang and Wood, 2011). Toxin-antitoxin systems are commonly carried on MGE including phages and plasmids to ensure maintenance of the element once it is integrated into the host (Yamaguchi et al., 2011). When co-expressed these components form a stable complex inhibiting the toxin's activity. Free antitoxin is highly unstable and degrades quickly requiring its continued synthesis to inhibit the toxin's function (Yamaguchi et al., 2011). Toxin-antitoxin systems are widespread in bacteria and are thought to promote cellular adaption through the regulation of cell growth. These elements can also regulate gene expression, control bacterial populations and contribute to programmed cell death and abortive infection (Yamaguchi et al., 2011),. a strategy used by bacteria to deter the spread of phages throughout the bacterial population. During phage infection, protein expression in the host can be slowed or arrested. If the infected host bacterium codes for a toxin-antitoxin system the liable antitoxin it produces will degrade, leaving the toxin to kill the cell, ultimately preventing the phage from replicating and spreading. The toxin-antitoxin systems identified here are mainly associated with Siphoviridae-like prophages, although two Myoviridae-like prophages (P10 and P11) contained a copy of the HipB antitoxin without its corresponding HipA toxin (Table 5.3). It is possible these are remnants of previously functioning toxin-antitoxin systems or are maintained by the phages as a means to avoid abortive infection by the host. This is a possibility given the HipA toxin gene was present in other regions of the chromosome of those *M. haemolytica* strains that contained both P10 and P11 phages.

Multiple virulence associated genes were present in the prophages identified, including a periplasmic chaperone LolA (P5), involved in outer membrane localization of lipoproteins, and Plp4 (P4), a lipoprotein with homology to OmpA. A peptidoglycan glycosyltransferase, MtgA (P5) with homology to penicillin binding protein was also present. A mutation in this gene in *Brucella abortus* was previously shown to attenuate virulence (Derouaux et al., 2008). SanA (P2) was also detected, which functions in murein synthesis, but also has been implicated in vancomycin resistance (Rida et al., 1996). A gene coding for a cytoplasmic protein UspA (P4, P14) was also present. Although the direct mechanisms by which this protein functions is unclear, it has been shown to promote cell survival during exposure to environmental stress (Siegele, 2005).

### 5.3.7 CRISPR-Cas

Nearly half of all bacterial genomes sequenced encoded for an adaptive immune system called CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRSIPR associated proteins) (Makarova et al., 2011; Szczepankowska, 2012). This system protects against invading genetic elements like phages and plasmids (Makarova et al., 2011). Three major types and ten subtypes of CRISPR-Cas systems have been identified (Makarova et al., 2011) all containing a CRISPR array typically localized near 4 to 20 CRISPR associated (Cas) genes. The CRISPR array consists of a leader sequence followed by numerous conserved direct repeat (DR) sequences, interspersed with variable spacer sequences (Szczepankowska, 2012). Spacers are typically derived from viruses or plasmids (Sampson et al., 2013; Samson et al., 2013; Szczepankowska, 2012) and are used by a suite of Cas proteins to target reinfection by these same viruses or plasmids through a process called interference (Makarova et al., 2011). Cas proteins support the integration of spacers into the CRISPR array, the processing of the transcribed CRISPR array into mature crRNA that contain a spacer and a partial DR, and the targeting and cleavage of reinvading nucleic acids complimentary to the spacer sequences (Makarova et al., 2011). The selection of proto-spacers (spacer precursors) from the invading nucleic acid is dependent on the presence of a proto-spacer-associated motif (PAM), which is a short nucleic acid sequence located either downstream or upstream of the proto-spacer. Proto-spacer-adjacent motifs are not incorporated into the spacer itself, enabling the CRISPR-Cas system to distinguish between target proto-spacer and the host CRISPR array (Szczepankowska, 2012). In addition to its role in defence CRISPR-Cas has also been implicated in gene expression and regulation of cellular processes including biofilm formation, lysogenization, spore formation, replicon maintenance and segregation, and DNA repair-recombination (Szczepankowska, 2012).

All of the *M. haemolytica* genomes contain a single type I-C/Dvulg CRISPR-Cas system (Figure 5.8A) (Makarova et al., 2011). These are highly conserved within serovar (>98% nucleotide identity) with the greatest sequence variation occurring between S2 and S1/S6 genomes, although overall pairwise identity across all strains was still >95% (Table S2). The leader and trailer sequences in all CRISPR arrays were highly conserved with >98% pairwise identify among strains (Figure 5.8B), a result that was expected as these regions are typically conserved within a species (Karginov and Hannon, 2010). Six different DR sequences of 32 nucleotides were identified in the CRISPR arrays, with variations in positions 1, 11, 22, 24 and/or 32 (Figure 5.8D). The CRISPR arrays are organized into two blocks in both the S1 and S6 genomes, each block consisting of a unique DR sequence followed by 4-8 identical DR sequences (Figure 5. 8C). In S2 strains, one block of DRs occurs and is identical to those in the first

block of S1/S6 arrays. Direct repeat sequences are target sites for Cas proteins (Stern et al., 2010) and sequence variation in these regions could affect the regulation of crRNA maturation.

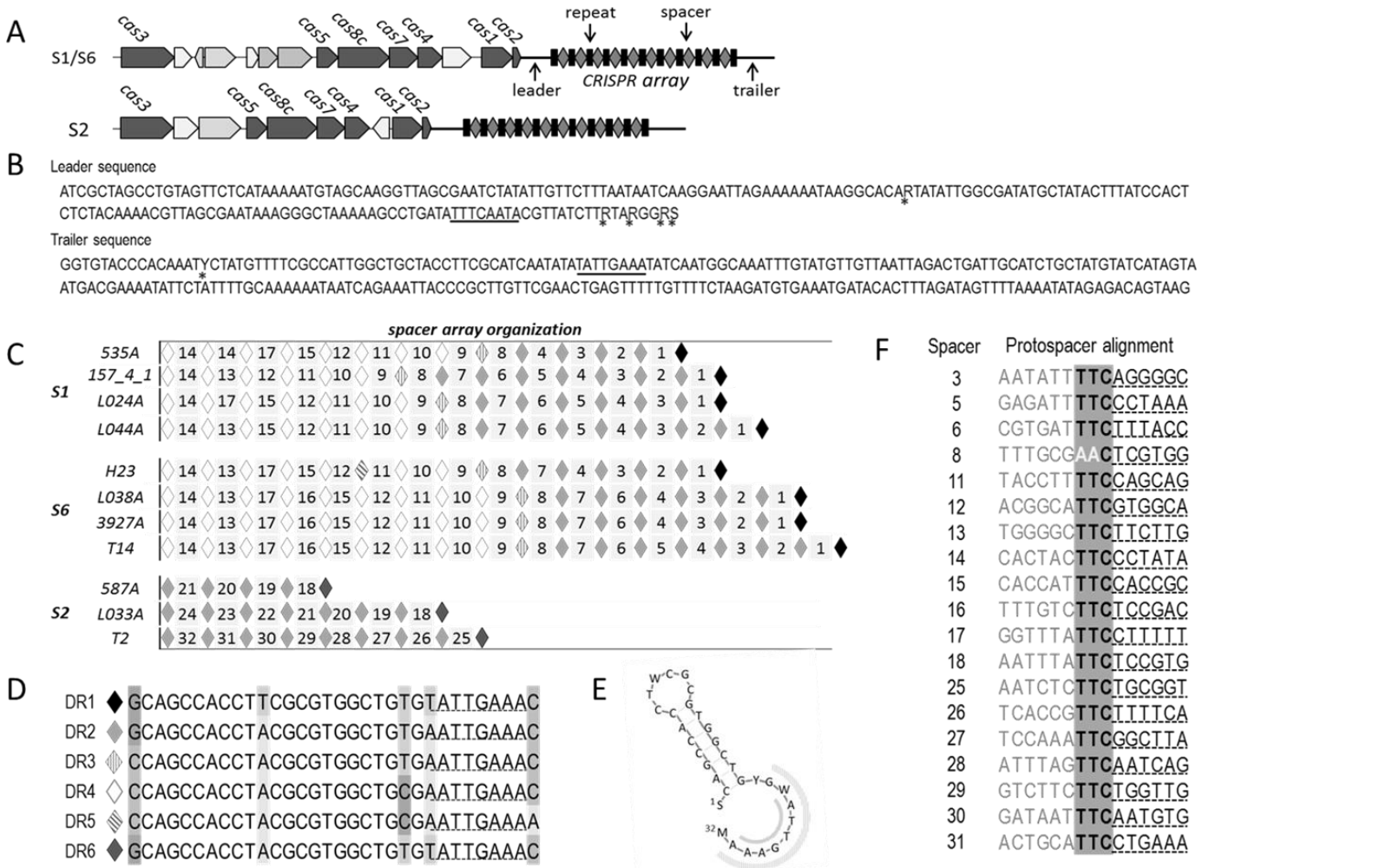


Figure 5.8 Schematic of CRISPR-Cas systems identified in whole genome sequence analysis of 11 *Mannheimia haemolytica genomes.* Panel A: Cas loci present in serotype 1,6 and 2 genomes. Panel B: consensus sequence for leader and trailer regions of CRISPR arrays. Underlined regions are complimentary to psi-tag. Panel C: CRISPR array spacer and direct repeat organization. Diamonds represent direct repeat sequences while numbered tiles represent spacer sequences. Panel D: Sequences of the 6 unique direct repeats detected in CRISPR arrays. Panel E: 3D structure of direct repeats found in CRISPR arrays. Psi-tag indicated by grey shaded regions. Panel F: sequence alignment of protospacers with suspected PAM highlighted in grey (TTC).

A conserved terminal eight nucleotide motif ((T/A)ATTGAAA) or psi-tag, implicated in Cas protein binding (Hale et al., 2009) and self-discrimination (Marraffini and Sontheimer, 2010), was identified in the DR sequences and in the leader (in reverse orientation) and the trailer of all CRISPR arrays (Figure 5.8E). The significance of this feature is unclear, but it could play a role in regulation of CRISPR activity. All DRs exhibit homology (90-100% nucleotide identify) to those occurring in arrays in other *Pasteurellaceae* members including *Bibersteinia trehalosi* USDA-ARS-USMARC-190, *Mannheimia varigena* USDA-ARS-USMARC-1312 and *Aggregatibacter actinomycetemcomitans* D7S-1 and have a secondary structure consistent with those from the CRISPR-Cas I-C/Dvulg subtype (Kunin et al., 2007) (Figure 5.8E).

Thirty-two unique spacer sequences (35-37 nt) were identified in the CRISPR arrays, with S2 genomes containing fewer spacers (4-8) than S1 (13-15) and S6 (14-17) (Figure 5.8C). A duplicate spacer was identified in the array of *M. haemolytica* 535A, an occurrence that has been previously identified (Jansen et al., 2002). The majority of the spacers are of phage origin, most corresponding to prophages present in the chromosome of the strains sequenced here (Table 5.4). It is possible that these CRISPR spacers are regulating lysogeny of the phages in these genomes; a phenomenon previously observed in *E. coli* (Edgar and Qimron, 2010).

Table 5.4 Spacer sequences identified in CRISPR arrays from 11 *M. haemolytica* genomes

| Spacer | No. of genomes with CRISPR spacer | No. of genomes with sequence outside of spacer | Spacer target[a] | Gene target |
|---|---|---|---|---|
| 1 | 8 | 0 | n/a | CRISPR |
| 2 | 6 | 0 | n/a | CRISPR |
| 3 | 6 | 6 | prophage | hypothetical |
| 4 | 8 | 0 | n/a | CRISPR |
| 5 | 8 | 4 | prophage | hypothetical |
| 6 | 8 | 8 | prophage | putative tail |
| 7 | 8 | 0 | n/a | CRISPR |
| 8 | 7 | 2 | prophage | regulatory protein Rha |
| 9 | 6 | 0 | n/a | CRISPR |
| 10 | 3 | 0 | n/a | CRISPR |
| 11 | 8 | 8 | prophage | hypothetical |
| 12 | 8 | 5 | prophage | hypothetical |
| 13 | 7 | 8 | bacterial | UDP-N-acetylglucosamine 2-epimerase |
| 14 | 8 | 5 | bacterial | glycosyl transferases group 1 |
| 15 | 6 | 7 | prophage | tail sheath protein FI |
| 16 | 3 | 4 | prophage | Mu protein F |
| 17 | 7 | 7 | prophage | hypothetical |
| 18 | 2 | 8 | prophage | hypothetical |
| 19 | 2 | 0 | n/a | CRISPR |
| 20 | 2 | 0 | n/a | CRISPR |
| 21 | 2 | 0 | n/a | CRISPR |
| 22 | 1 | 0 | n/a | CRISPR |
| 23 | 1 | 0 | n/a | CRISPR |
| 24 | 1 | 0 | n/a | CRISPR |
| 25 | 1 | 7 | prophage | DNA helicase |
| 26 | 1 | 6 | prophage | hypothetical |
| 27 | 1 | 8 | prophage | head morphogenesis protein |
| 28 | 1 | 10 | prophage | hypothetical |
| 29 | 1 | 10 | prophage | hypothetical |
| 30 | 1 | 1 | prophage | pyruvate kinase |
| 31 | 1 | 15 | prophage | hypothetical |
| 32 | 1 | 0 | n/a | CRISPR |

[a] n/a, spacer sequence not identified in prophage located in host chromosome

Bacteriophages have developed multiple strategies to avoid CRISPR regulation, including mutations within either the proto-spacer or PAM or through phage encoded anti-CRISPR systems (Peng et al., 2003; Sorek et al., 2008). As a result, it is not surprising to see multiple prophages integrated into the S1 and S6 genomes even though these strains contain large CRISPR arrays. Homology searches of the prophage regions here failed to identify any previously described phage encoded anti- CRISPR

systems (Peng et al., 2003; Sorek et al., 2008). Consistent with other type I-C CRSIPR-Cas (Kupczok and Bollback, 2014), examination of the upstream sequences of the proto-spacers revealed a 3nt PAM of GAA conserved in all but one target proto-spacer sequence (Figure 5.8F).

Approximately 18% of all CRISPR bearing organisms contained self-targeting spacers (Stern et al., 2010). However, these spacers lack conservation, meaning they don't typically occur in more than one strain or isolate, and they are often associated with CRISPR systems that have partially or fully degraded activity (Stern et al., 2010). An exception to this occurs in the intracellular pathogen *Francisella novicida*, where a self-targeting CRISPR-Cas system facilitates immune evasion during infection (Sampson et al., 2013). In this species the CRISPR system suppresses expression of an immune stimulatory lipoprotein (BLP) that would otherwise trigger the activation of Toll-like Receptor 2-dependant proinflammatory response in the host during infection. Absence of this regulation completely attenuates the bacteria during infection making CRISPR-Cas critical for *F. novicida* pathogenesis (Sampson et al., 2013).

Two spacers present in S1 and/or S6 but not in S2 CRISPR arrays were complimentary to sequences encoded on the host chromosome; one for a glycosyl transferases group 1 domain (F388_04799), and a second for a UDP-N-acetylglucosamine 2-epimerase (F388_04774). Glycosyl transferases transfer activated sugars to a variety of substrates and UDP-N-acetylglucosamine 2-epimerase is a rate limiting enzyme in the sialic acid biosynthetic pathway. Sialic acids are nine-carbon-sugars prevalent on the surface of vertebrate cells that serve a variety of biological, biophysical and immunological functions (Lewis and Lewis, 2012). As a result many pathogens, including *Neisseria meningitidis*, *Campylobacter jejuni*, Group B *Streptococcus*, *Pseudomonas aeruginosa* and *Haemophilus influenza* (Angata and Varki, 2000), incorporate sialic acid on their cell surface as a form of molecular mimicry to avoid immune detection in the host or to aid in adhesion (Lewis and Lewis, 2012; Severi et al., 2007). Given the CRISPR systems here appear intact it is possible that like *F. novicida*, *M. haemolytica* employs CRISPR-Cas to regulate gene expression of proteins that control the display of sugar moieties and sialic acid on the bacterial cell surface. This would possibly contribute to immune subversion or enhanced colonization during infection as spacers against these genes were not identified in commensal S2 strains.

**5.3.8 Integrative conjugative elements**

Putative ICEs were identified in 9 of the 11 genomes sequenced, ranging in in size from 46.6 to 81.1 kb (Figure 5.9A). These elements are self-transmissible MGE that encode all of the necessary machinery for replication and conjugative transfer (Jackson et al., 2011; Wozniak and Waldor, 2010).

Integrative conjugative elements are integrated into the host genome, but under certain conditions can excise, circularize and replicate. Transfer of ICEs in Gram-negative bacteria typically occurs through conjugation machinery coded for by a type 4 secretion system (T4SS), a system similar to that coded for on conjugative plasmids, consisting of a membrane spanning secretion channel and extracellular pilus (Wozniak and Waldor, 2010). After an element has excised from the chromosome, a relaxosome formed by several proteins assembles at the origin of transfer. A relaxase nicks the double stranded circular element and then while maintaining a complex with the single stranded DNA is recognized by a coupling protein and recruited to the T4SS translocation channel. After the DNA is translocated, the relaxase detaches and the element is circularized. Complimentary DNA is synthesized and the double stranded element is integrated into the chromosome by an integrase (Juhas, 2015). A copy of the original ICE remains in the donor and undergoes complimentary strand synthesis and reintegrates back into the host genome (Wozniak and Waldor, 2010). Indeed, all the ICEs identified here were integrated into the same copy of the $tRNA^{Leu}$ gene and contained a type IV secretion system (T4SS), a coupling protein, an integrase and a relaxase (Figure 5.9; Table A2 in Appendix A), (Guglielmini et al., 2014).

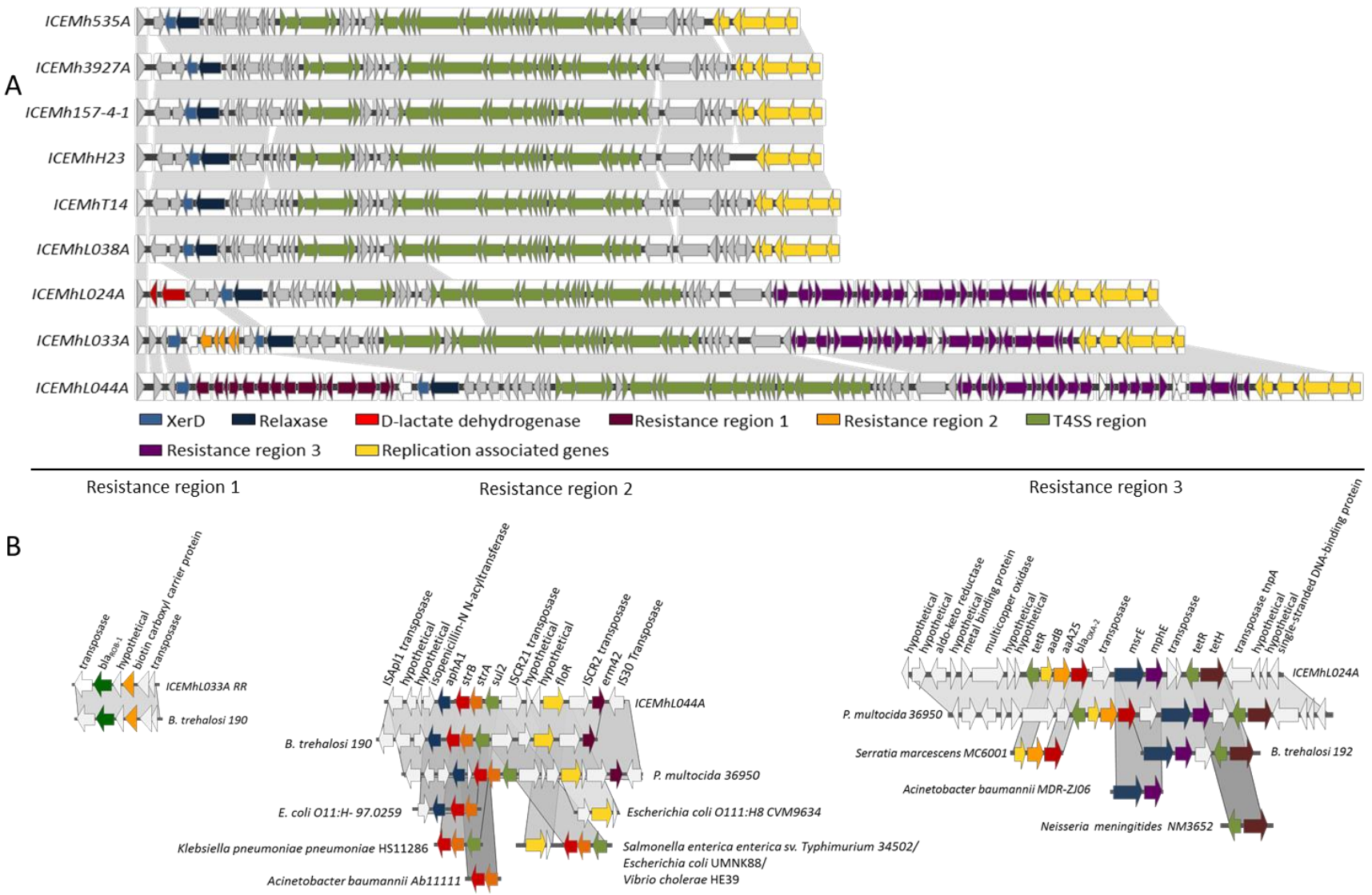


Figure 5.9 Schematic of putative integrative conjugative elements identified in whole genome sequences of *Mannheimia haemolytica*. Genes are represented as arrows. Grey background indicated regions >94.5% sequence identity. Panel A: proposed ICE genes arrangement. Panel B: resistance gene regions with alignments against cassettes found in other bacterial species.

All T4SSs identified belonged to the $MPF_G$ family, originally described in ICEHin1056 from *Haemophilus influenzae* (Guglielmini et al., 2014), but prevalent in *Pasteurellaceae* (*Gallibacterium anatis*, *Pasteurella multocida* and *Histophilus somni*) and various Gammaproteobacteria (V*ibrio cholera, E. coli, Pseudomonas aeruginosa, Klebsiella pneumonia and Salmonella enterica).* Three distinct sequence profiles for the T4SS region were identified with an overall nucleotide similarity of 85.8%, indicating that the ICEs did not all originate from the same source. However, 6 of the 9 ICEs had a nucleotide similarity of 95.5% across the entire array. This, coupled with their prevalence across genetically diverse isolates indicates that ICEs may have an ancient lineage within this species. None of these 'common' ICE profiles contained accessory gene cassettes so it is unlikely that they are being selected for by antimicrobials. However, contig alignment against the previously identified ICE*Mh*1 indicates that they may contain a HipA/B toxin/anti-toxin system. Closure of these genomes would be required to conclusively determine if this system is contained within these elements, but the presence of toxin-antitoxin systems could explain why ICEs are being maintained in the *M. haemolytica* population in such high abundance. This observation would indicate that these elements would not have to necessarily contain genes coding for antimicrobial resistance (AMR) in order for selective pressure to ensure their retention within the genome.

Entry exclusion prevents conjugative transfer of duplicate elements with closely related transfer apparatuses into bacterial cells (Thomas and Nielsen, 2005) and is system characteristic of conjugative plasmids (Garcillán-Barcia and de la Cruz, 2008). Two mechanisms for entry exclusion in F plasmids have been identified, one that alters the cell's outer surface, making it less receptive to pilus attachment and the second which is mediated by proteins that sit in the inner membrane and prevent DNA entry (Marrero and Waldor, 2007a, b; Thomas and Nielsen, 2005). An exclusion system has been well characterized in the SXT/R391 family of ICEs and is mediated by small inner membrane proteins expressed by both the donor (TraG) and recipient cells (Eex) (Marrero and Waldor, 2007a). We annotated a TraG homolog in the T4SSs here (F388_01689) a gene that exhibited >80% nucleotide similarity to that in ICEs from other *Pasteurellaceae* species (*Mannheimia varigena* USDA-ARS-USMARC-1388:CP006953, *Bibersteinia trehalosi* USDA-ARS-USMARC-190:CP006956*, Pasteurella multocida* 36590:CP003022, *Actinobacillus pleuropneumoniae*; NZ_ADX001000031, *Actinobacillus suis* ATCC 33415:Ga0057047_gi672591300.1, *Haemophilus parasuis* D74:Ga0040714_123, *Haemophilus somnus* 2336:NC_010519). A potential analog to Eex was also identified in our work (F388_01694) and in the genomes of the species listed above. This coding sequence displays characteristics that are similar to Eex in SXT/R391; being cytoplasmic, located in the opposite orientation but adjacent to the TraG gene

and displaying significant AA similarity in the N terminal region, but a high degree of diversity in the C-terminal region (Marrero and Waldor, 2007b).

Only three of the ICEs, ICEMhL024A, ICEMhL044A and ICEMhL033A, contained cassettes with accessory genes but those that did possessed genes coding for AMR. These cassettes were highly similar to those previously identified in *Pasteurella multocida* (PRJNA86887) and *Bibersteinia trehalosi* 190 (GCA_000521765.1) with portions of these cassette or individual genes also being identified in strains of *E. coli*, *Klebsiella pnuemoniae*, *Salmonella*, *Actinobacter baumannii*, *Serratia marcescens* and *Neisseria meningitides* (Figure 5.9B). In addition to multiple AMR genes including; *aphA1, strB, strA, sul2, floR, erm42, tetH, aadB, aaA25, msrE, and mhpE*, a $bla_{ROB-1}$ was also identified in ICEMhL033A with a single nucleotide substitution from C to T at position 370. This substitution generated a premature stop codon, rendering this gene non-functional in making these strains susceptible to these antimicrobials (as presented in Chapter 3).

Although only one of the three S2 strains examined here contained ICE, it harboured an extensive AMR profile. As S2 strains are prevalent commensals among healthy cattle the spread of AMR ICEs among these populations may be more significant to the environmental resistome than AMR ICE in S1 and S6 strains that may be removed from the population through effective drug therapies or if the host animal succumbs to disease. All of the AMR containing ICEs originated from strains of *M. haemolytica* isolated from cattle in the USA. Phylogenetic analysis of a larger population incorporating different *Pasteurellaceae* species from different animal production systems would be of benefit to determine the origin of these elements. Source tracking of isolates is a common practice to gain insight into AMR development and spread, but with the mobility of those elements between species it may become just as important in the future to track ICEs between and within production systems rather than focusing on AMR at the species or strain level.

The potential for entry exclusion in ICEs from *M. haemolytica* may affect AMR dissemination. If the absence of ICEs resistance cassettes is widespread among *M. haemolytica* populations, entry exclusion could render these strains resistant to the uptake of ICEs from other members within the population that may contain AMR genes. This would limit ICE spread primarily to clonal dissemination in *M. haemolytica* populations or to those cases where the entry exclusion system was ineffective. If non-AMR ICEs are as prevalent in the *Pasteurellaceae* family as they appear based on this dataset, entry exclusion by these elements could explain why there is relatively a low overall occurrence of AMR in members the *Pasteurellaceae* family (Klima et al., 2011; Watts and Sweeney, 2010).

A D-lactate dehydrogenase was identified in ICEMhL024A that is also present in the chromosome of various *Pasteurellaceae* species (*Bibersteinia trehalosi*: USDA-ARS-USMARC-190, USDA-ARS-USMARC-192, USDA-ARS-USMARC-188*; Haemophilus parasuis*: 84-17975, KL0318; *Haemophilus somni* 129PT; *Actinobacillus pleuropneumoniae*: JL03, AP76, SH0165) including the ICEs in multiple genomes of *M. haemolytica* (*Mannheimia haemolytica*: M4258, USDA-ARS-USMARC-185, USDA-ARS-USMARC-185, D153). Lactate is a by-product of bacterial fermentation. The L-form of this carboxylic acid is readily metabolized in the liver and tissues of mammals, but high levels of D lactate can accumulate in the blood of cattle during acidosis, a condition common in feedlot calves feed high grain finishing diets (Nagaraja et al., 1981). It is possible that D-lactate dehydrogenase is being used by *M. haemolytica* to metabolize the D isomer of lactate as an energy source.

A multicopper oxidase was also identified in resistance region three of all three ICEs exhibiting AMR (Figure 5.9B). Multiple roles have been proposed for multicopper oxidases that include iron transport and copper resistance (Kellner et al., 2008). Copper is a trace element added to cattle feed (Larney et al., 2014), the vast majority of which, as with all other heavy metals, is excreted in faeces and urine (Petersen et al., 2007). It is possible the multicopper oxidase here is playing a role in copper resistance as a result of environmental exposure. It is also conceivable that the gene is functioning in iron sequestration, a function linked to the pathogenesis of *M. haemolytica* (Sathiamoorthy et al., 2011).

**5.4 Conclusion**

Multiple factors contribute to virulence in *M. haemolytica*. Comparative analysis of whole genome sequences of pathogenic and non-pathogenic serovars has highlighted sequence diversity and functional differences in key virulence factors in particular iron acquisition and outer membrane proteins. Overall, S2 genomes contained fewer intact prophages. These elements likely contribute to virulence profiles in S1 and S6 strains as some of these phages were shown to harbour virulence-associated genes. It is also possible that CRISPR-Cas is playing a role in the surface expression of sialic acid residues on the surface of S1 and S6 strains contributing to immune evasion during infection. Integrative conjugative elements were found in all but two strains, both of which were serotype2, and are likely playing a role in enhancing host survival in strains that harboured multidrug resistant cassettes. It is possible that these elements are regulating their own dissemination through populations through entry exclusion. This holds implication for spread of AMR within *M. haemolytica* as well as possibly other bacterial populations as a result of the transfer of these mobile genetic elements within bacterial populations.

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# 6 Chapter 6: *In silico* identification and high throughput screening of antigenic proteins as candidates for a *Mannheimia haemolytica* vaccine

Chapter 6 is not intended for publication.

This manuscript was drafted by Cassidy Klima with suggestions and comments by Dr. Tim McAllister, Dr. Rahat Zaheer, and Shaun Cook. Experimental design, data collection and analysis were undertaken by Cassidy Klima and Dr. Rahat Zaheer.

**6.1 Introduction**

Vaccination is one of the most efficient and cost-effective management strategies to control and eliminate disease (Rappuoli and Aderem, 2011). With the significant advancements in "omics" technologies over the past two decades, the approaches used for vaccine design have also evolved. Traditional methods used to generate first and second generation vaccines have focused on the whole organism based on the principals of "isolate, inactive and inject" proposed by Pasteur in the 19th century (Doolan et al., 2014). The availability of whole genome sequencing has shifted the focus towards *in silico* antigen prediction for the development of third generation vaccines. This reverse vaccinology (RV) approach starts from the genome rather than the whole organism and identifies the entire catalog of proteins that an organism has the potential to express at any point in time. This strategy has the added benefit of being applicable to cultivatable and non-cultivatable species (Rappuoli, 2001).

*In silico* prediction of vaccine candidates relies on software that can screen whole genome sequences and identify coding DNA. The subcellular localization of these sequences is determined by bioinformatics tools that identify signatures like signal peptides, transmembrane helices or cell-wall anchor motifs associated with surface exposed or secreted proteins (Bertholet et al., 2014). These proteins are most likely to be accessible to the immune system and thus make good vaccine targets. Comparative genomics and pan-genome analysis can be coupled with this strategy to ensure the antigens selected provide maximum coverage for a universal vaccine against a diverse species or to avoid cross reactivity against non-target species like commensal organisms (Moriel et al., 2008).

The strategy to start from the genome rather than the organism was first proposed by Rappuoli (2000). Since the conception of RV, progress in genomic, proteome, and transcriptome analysis have had a massive impact on the speed that novel antigens are being identified (Rappuoli and Aderem, 2011). However, challenges still exist with evaluating the antigenicity of a large number of vaccine candidates. Traditional antigen production strategies require the cloning and expression of several hundred proteins (Pizza et al., 2000) and their purification prior to evaluation in immunogenicity assays. These methods are often laborious and challenging due to the many complications associated with expression of outer membrane proteins including issues of low solubility, the formation of inclusion bodies, protein instabilities and toxicity to the expression host (Rosenblum and Cooperman, 2014).

This study employed a RV approach to identify conserved antigens in pathogenic strains of *M. haemolytica* for the purpose of developing of a bovine vaccine against this organism. A comparative genomics approach was taken to identify candidates in the pan-genome that provide broad protection

against serotypes 1, 2 and 6 of this pathogen. To screen antigen candidates in a high throughput manner, this study utilized the unique application of a cell-free translation system coupled with ELISA to screen proteins for their immunoreactivity against sera generated n cattle experimentally infected with *M. haemolytica*. This methodology drastically reduced the time required to screen hundreds of antigen candidates for immunoreactivity, over the span of weeks as compared to the months that would be required to clone and express individual candidate proteins within a host expression system.

## 6.2 Material and methods

### 6.2.1 Antigen candidate selection

Pan-genome analysis was used to identify groups of coding genes present in the whole genome sequence of 23 *M. haemolytica* isolates (Table 6.1). The analysis, performed using a pan-genome analysis pipeline (PGAP) (Zhao et al., 2012), produced a gene cluster file that grouped all orthologues present in the dataset based on cut-off values set at 95% sequence identity over 90% of the sequence length. From this file, orthologous clusters were selected based on the gene occurrence in at least 50% of genomes. A single representative gene from each cluster was selected, prioritizing the *M. haemolytica* L044A genome. The *M. haemolytica* L044A genome was given priority due to its large size among the sequenced genomes, anticipating its use as a template for amplifying the majority of target genes for downstream expression and immunoreactivity studies. If the representative gene was absent in *M. haemolytica* L044A, it was selected from the genomes of strains from serotypes 1 or 6 including *M. haemolytica* L024A, L038A or H23. The resulting group of genes was screened using SignalP (Petersen et al., 2011) to identify N-terminal signal peptides, indicative of gene products that are secreted from the cell. The TMHMM server v 2.0 was also used to identify proteins containing transmembrane helices that are typically associated with the cell membrane (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The program PSORTb 3.0 (Nancy et al., 2010) was used to predict the localization of each gene product within the cell. Those candidates identified as having N-terminal signal peptides, with fewer than 4 transmembrane helices, with known or predicted localization to either the periplasm, cell membrane, extracellular secretion, identified as lipoproteins, or predicted hypothetical proteins with these desirable features were further examined in an immunoreactivity assay using a coupled-cell free transcription/translation system for protein expression.

Table 6.1 Strains of *Mannheimia haemolytica* used in pan-genome analysis

| Strain | Accession Number | Serovar | Isolate origin |
|---|---|---|---|
| *M. haemolytica* L024A | n/a | 1 | Texas |
| *M. haemolytica* L044A | n/a | 1 | Nebraska |
| *M. haemolytica* 157-4-1 | n/a | 1 | Alberta |
| *M. haemolytica* 535A | n/a | 1 | Alberta |
| *M. haemolytica* T2 | n/a | 2 | France |
| *M. haemolytica* L033A | n/a | 2 | Nebraska |
| *M. haemolytica* 587A | n/a | 2 | Alberta |
| *M. haemolytica* L038A | n/a | 6 | Alberta |
| *M. haemolytica* T14 | n/a | 6 | France |
| *M. haemolytica* H23 | n/a | 6 | Alberta |
| *M. haemolytica* 3927A | n/a | 6 | Alberta |
| *M. haemolytica* USDA-ARS-USMARC 2286 | NC_021883.1 | n/a | Nebraska |
| *M. haemolytica* D174 | NC_021739.1 | 6 | Iowa |
| *M. haemolytica* D171 | NC_021738.1 | 2 | Iowa |
| *M. haemolytica* D153 | NC_021743.1 | 1 | Iowa |
| *M. haemolytica* MhSwine2000 | ATTA00000000.1 | 1 | Iowa |
| *M. haemolytica* MhBrain2012 | ATSZ00000000.1 | 1 | Georgia |
| *M. haemolytica* D38 | AUNL00000000.1 | 6 | Iowa |
| *M. haemolytica* D35 | AUNK00000000.1 | 2 | Iowa |
| *M. haemolytica* D193 | ATSY00000000.1 | 1 | Iowa |
| *M. haemolytica* M42548 | NC_021082.1 | 1 | Pennsylvania |
| *M. haemolytica* USDA-ARS-USMARC-183 | NC_020833.1 | 1 | Kansas |
| *M. haemolytica* USDA-ARS-USMARC-185 | NC_020834.1 | 6 | Kansas |
| *M. haemolytica* PHL213 | NZ_AASA00000000.1 | 1 | n/a |
| *M. haemolytica* Bovine A2 | NZ_ACZY00000000.1 | 2 | n/a |
| *M. haemolytica* Ovine A2 | NZ_ACZX00000000.1 | 2 | n/a |

### 6.2.2 Protein expression

The EasyXpress Protein Synthesis kit (RiNA GmbH, Germany) was used to express genes encoding for protein candidates *in vitro* according to manufactures specifications. Template for the PCR reactions use to generate selected genes originated from overnight cultures of *M. haemolytica* L044A, *M. haemolytica* L024A, *M. haemolytica* L038A or *M. haemolytica* H23 grown of tryptic soy agar (TSA) blood at 37°C. The DNA was isolated using phenol: chloroform extraction method, as described in chapter 5.

According to the EasyExpress Liner Template kit instructions, a two-stage PCR process was used to generate a transcriptionally active PCR product. This product contained the coding sequence of the targeted antigen along with the regulatory elements required for optimal transcription and translation in a cell-free expression system and an N-terminal *Strep*-tag, and a C-terminal 6xHis tag. The first PCR used primers generated specifically for each coding sequence with an additional 5' adaptor consisting of 30 nt for the forward primer and 20 nt for the reverse. These 5' adaptors were targets for the second round of PCR that was used to add the regulatory elements and end terminal tags to the final PCR product. For the first round of PCR a two-step program was used. After an activation step of 95°C for 30 s, 5 cycles of 94°C for 1 min, 55° for 1 min and 72°C for 2 min were performed followed by 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min, followed by a final 10 min extension at 72°C. A list of the gene targets, their characteristics and primers used is presented in Table A3 in Appendix A. The second round of PCR used amplicon products from the first PCR as template according to manufacturer's specifications. A two-step program was also used for this second round of PCR. After an activation step of 95°C for 30 s, 5 cycles of 94°C for 1 min, 50° for 1 min and 72°C for 2 min were performed followed by 35 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2 min, followed by a final 10 min extension at 72°C. The HotStar HiFidelity Polymerase Kit (Qiagen Canada Inc., Toronto, ON) was used for both rounds of PCR reactions.

The EasyXpress Protein Synthesis kit was used according to manufacturer's specifications to express the tagged proteins used for immunoreactive screening. A 40 µL reaction volume was set up for each targeted protein expression using 5.6 µL of the final product from the final PCR reaction as expression template. The expression reactions were incubated in a thermo-mixer at 33°C for 90 min at 300 rpm.

### 6.2.3 Generation of sera against *M. haemolytica* for antigen screening

To generate the sera for screening antigenic candidates, three groups of three calves each were challenged twice, on day 1 and 28, with intranasal challenge of either serotype 1, 2 or 6 strains of *M. haemolytica*. The sequences used were the ones sequenced during the present study and used to infer pan genome. The serotype 1 group inoculum consisted of a mixture of *M. haemolytica* L024A, *M. haemolytica* L044A, *M. haemolytica* 535A and *M. haemolytica* 157-4-1. The serotype 2 group inoculum consisted of a mixture of *M. haemolytica* 587A, *M. haemolytica* L033A, and *M. haemolytica* T2. And the serotype 6 group inoculum consisted of a mixture of *M. haemolytica* L038A, *M. haemolytica* H23, *M. haemolytica* T14, and *M. haemolytica* 3927A.

To prepare bacteria for intranasal challenge, 350 mL aliquots of brain heart infusion (BHI) broth were each inoculated at 1:100 dilution of overnight cultures *M. haemolytica* strains. The cultures were grown at 37°C to an optical density at 600 nm between 0.6 – 0.8. Cultures of like serotypes were pooled using equal volumes and the colony forming units (cfu) of resulting suspensions were adjusted to 7.9 – 6.4 x $10^9$ cfu/mL. A 25 mL aliquot of each pooled cell suspension was used for intratracheal administration to the animals. The second challenge was prepared the same as the first except the administered dosages were lower ranging between 2.7 – 3.7 x $10^9$ cfu/mL. Blood samples were collected on day 42 and processed for sera isolation as previously described (Harland *et al.,* 1992). Serum samples were stored at -80°C. For immunoreactivity screening, equivalent volumes of sera from each of the animals inoculated with each specific serotype strains of *M. haemolytica* were pooled. Throughout the study animals were handled in accordance with the guidelines set by the Canadian Council on animal care (http://www.ccac.ca/).

**6.2.4 Enzyme-linked immunosorbent assay to determine immunoreactivity of sera**

Proteins were screened with ELISA, in parallel for their relative quantity and immunoreactivity with polyclonal antisera raised in calves experimentally infected with *M. haemolytica*. For the ELISAs 360 µL of solubilisation buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole and 1.2% Triton X-100) was added to 40 µL of the translated protein product and mixed. One hundred microliters of solubilized protein mixture was added into each of four wells in $Ni^{+2}$ coated plates (Pierce, Cat # 15142 or 15442) and incubated for 1 h at room temperature with shaking at 400 rpm in a plate thermomixer (Eppendorf Canada Inc., Mississauga, ON). The mixture containing the unbound proteins was removed and the wells were washed 3 times for 5 min each, with 200 µL of wash buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 30 mM imidazole). Strep-tactin-HRP conjugate (150 µL; product # 161-0382; Bio-Rad Laboratories, Inc. Hercules, CA, USA) was added to one set of wells (Strep-ELISA plate) and diluted 1:10,000 using antibody dilution buffer (phosphate buffered saline (PBS) containing 0.05% Tween-20 and 2% bovine serum albumin). The Strep-ELISA plates were incubated for 1 h at room temperature with shaking at 400 rpm. The polyclonal antibodies raised in calves against serotypes 1, 2 or 6 of *M. haemolytica* were each added at 1:500 dilution in antibody dilution buffer to the $Ni^{+2}$ bound proteins in one of the remaining three plates (anti-S1 sera-ELISA plate; anti-S2-ELISA sera plate; anti-S6-ELISA sera plate) and incubated for 1 h at room temperature with shaking at 400 rpm. Plates were washed 3 times for 5 min each using 200 µL of ELISA wash buffer (PBS containing 0.05% Tween-20). The Strep-ELISA plates were processed for detection using SIGMAFAST™ OPD tablets (P9187, Sigma-Aldrich, Oakville, ON, Canada) according to manufacturer's instructions. To the anti-S1 ,

anti-S2 and anti-S6 sera-ELISA plates, goat anti-bovine IgG-HRP antibody was diluted 1:30,000 in dilution buffer (200 µL) and incubated at room temperature for 1 h with shaking at 400 rpm. The resultant mixture was washed 3 times, for 5 min each with 200 µL of ELISA wash buffer. The anti-S1, anti-S2 and anti-S6 sera-ELISA plates were processed for detection as described above. All assays were performed in duplicate. The data from all plates was normalized by subtracting the corresponding blank samples. Relative immunoreactivity was calculated as the ratio of OD between test and control well standardized against the highly expressing control protein PlpE.

**6.3 Results**

Pan-genome analysis identified 11,538 orthologous clusters of genes across the 23 analyzed *M. haemolytica* genomes. A total of 2,341genes were present in at least half of the genomes and used for further analysis. From the list of 2341 genes, a total of 240 candidates were identified by SignalP and PSORT containing N-terminal signal peptides with diverse sub-cellular localizations (78 periplasmic, 52 outer membrane, 15 extracellular, 13 cytoplasmic membrane and 82 unknown). For ease of use in the expression system, some (n=43) genes were truncated into equivalently sized products (size range ≤ 1kb) resulting in a total of 291 candidates for screening for immunoreactivity with bovine sera. A total of 246 of the 291 proteins expressed and were captured so as to be measurable using the Strep-ELISA plates. In total, 186 candidate proteins were immunoreactive to at least one type of sera tested; 58 periplasmic, 45 outer membrane, 12 extracellular, 10 cytoplasmic membranes and 61 unknowns. Of these screened candidates, 105 were immunoreactive to all sera screened; 32 periplasmic proteins, 26 outer membrane proteins, 6 extracellular proteins, 7 cytoplasmic membrane proteins and 34 unknown proteins. The top ten antigens ranked based on immunoreactivity are shown in Figure 6.1 and include the serine protease Ssa-1 (MhL044A_02282), competence protein ComE (MhL044A_02398), a solute binding protein (MhL044A_00030), an outer membrane protein (MhL044A_02240), a molybdenum ABC transporter (MhL044A_02132), an ABC (ATP-binding cassette)-type dipeptide transport system (MhL044A_00839), a filamentous hemagglutinin (MhL044A_00334_402H), a conserved hypothetical protein (MhL044A_01577), a chaperone for outer membrane protein SurA (MhL044A_00827) and a porin protein (MhL024A_01035_563B). The overall rankings of the proteins based on ELISA, broken down by the serum used and by the overall average of relative immunoreactivity are presented in Table A3 in Appendix A.

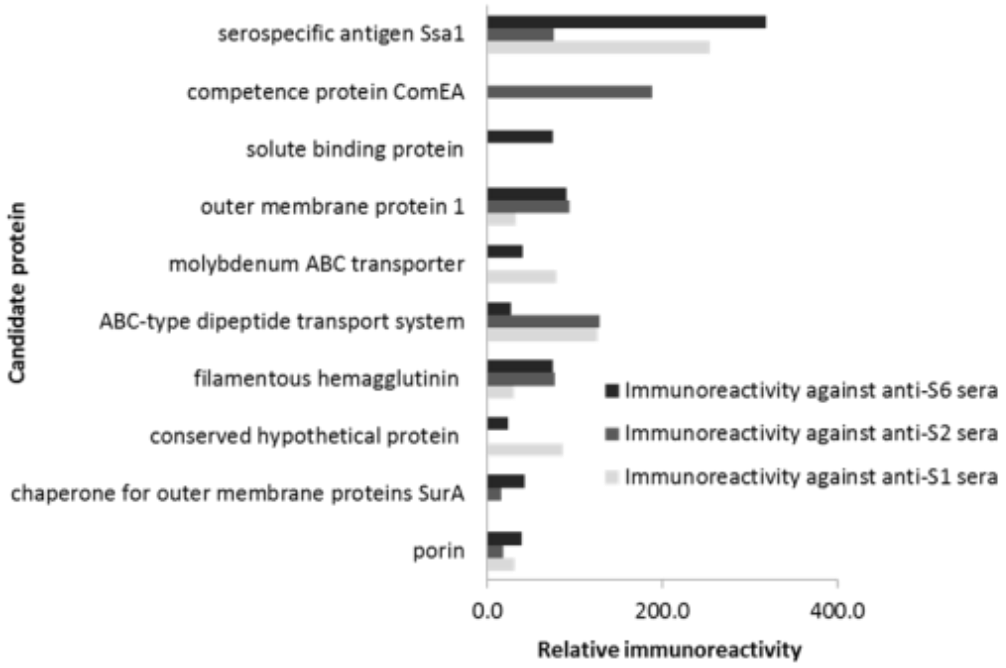


Figure 6.1 Top ranked antigen candidates based on serological screening. Relative immunoreactivity was calculated as the ratio of OD between test and control well standardized against the control protein PlpE.

**6.4 Discussion**

Of the top ten ranking candidates identified, the first five are discussed in further detail. The highest ranking candidate in the immunoreactivity assay was the serotype-1 specific antigen, Ssa1. This protein has been implicated in colonization of the nasopharynx (Highlander, 2001) and likely has protease activity (Sathiamoorthy et al., 2011). Although shown to elicit a strong antibody response in both rabbits and cattle (Lo, 2001) it has also been shown to be down-regulated 27-fold during infection *in vivo* (Sathiamoorthy et al., 2011). Ssa1 has previously been examined as a vaccine candidate against *M. haemolytica* (Ayalew et al., 2011) and has recently been found to rank within the top 10 of 55 vaccine candidates identified through two-dimensional polyacrylamide gel-based immunoproteomic analyses of *M. haemolytica* outer membrane vesicles (Ayalew et al., 2010). Although previously only shown to express *in vitro* in S1 strains of *M .haemolytica* (Highlander, 2001) the data here suggests that the antigen can also elicit an antibody response *in vivo* from S6 strains and to a lesser extent S2 strains.

The second highest ranking candidate was identified as the competence protein ComE. Competence is the mechanism by which bacteria acquire exogenous DNA through transformation (Chen and Dubnau, 2004) and is a feature common to both Gram-positive and Gram-negative bacteria

(Redfield et al., 2006). Homologs of ComE have been found in all members of the *Pasteurellaceae* family functioning in fibronectin adhesion, DNA adhesion and in natural transformation (Mullen et al., 2008). Although ComE was predicted by PSORT to be localized to the cytoplasmic membrane, the homolog ComEA in *P. multocida* was shown by transmission electron microscopy to be localized to the bacterial surface (Hatfaludi et al., 2010). Incidentally, *P. multocida* ComEA (PMCN03_2052) expresses 43.5% pairwise identity with ComE from *M. haemolytica* and also localizes to the cytoplasmic membrane.

The third highest ranked protein was predicted to be a solute binding protein with PSI-BLAST identifying it as the periplasmic component of a maltose ABC (ATP-binding cassette) transporter. The ABC transporters have a diverse range of functions in bacteria including nutrient uptake and drug resistance, but are also associated with virulence through metal ion acquisition and the facilitation of bacterial attachment (Garmory and Titball, 2004). Protein components of ABC transport systems have been previously shown to be immunogenic against bacteria using mouse models (Garmory and Titball, 2004) with vaccines targeting ABC systems in *Mycobacterium tuberculosis* (Briles et al., 2000) and *Streptococcus pneumonia* (Tanghe et al., 1999) being developed in this manner. Interestingly, the fifth highest ranking candidate protein was also the periplasmic component of an ABC transporter, but one specified for molybdenum transport. The components of ABC transporters that are expected to be the most immunogenic are the outer membrane proteins as they are anticipated to interface with the host immune system. However, given that two of the top five antigen candidates here were identified as periplasmic components, it may be important to include proteins localized to this cellular compartment in vaccine design when applying RV. It is possible that because these proteins are not readily accessible to the immune system, they are not targets for opsonization and although clearly able to be displayed by antigen presenting cell resulting in an antibody response, they may not in fact be protective during challenge trials

The fourth highest ranked candidate in this assay was identified as an unknown outer membrane protein. No further information could be determined about its function although it is also found in *Mannheimia varigena* and *Bibersteinia trehalosi*, which are also members of the *Pasteurellaceae* family. This finding is particularly interesting, as it highlights the advantages of using RV to identify candidates that may potentially offer protection against multiple species of respiratory pathogens.

Multiple antigens that have been previously identified as candidates for the development of vaccines against *M. haemolytica,* but did not rank within the top 10 candidates were identified.. These include leukotoxin (Ayalew et al., 2009; Lee et al., 2001), the GS60 antigen that belongs to the LppC

family of outer membrane lipoproteins (Lee et al., 2008), the lipoprotein PlpE (Ayalew et al., 2009), the outer membrane proteins OmpA (Ayalew et al., 2011), and the transferrin-binding proteins TbpA and TbpB (Potter et al., 1999). The LktA protein (MhL044A_00993) did not express or bind with sufficient affinity to for its use in this ELISA assay so it was not evaluated further. The overall average immunoreactivity for PlpE (MhL044A_02777) was low, ranking at 95$^{tht}$ and the OmpA protein (MhL044A_01473) ranked 108$^{th}$. In theory, cell free systems contain all of the necessary components for transcription, translation and protein folding including ribosomes, aminoacyl-tRNA synthetases, translation initiation and elongation factors, ribosome release factors, nucleotide recycling enzymes, metabolic enzymes, chaperones, and foldases (Carlson et al., 2012). However, cell free systems are limited in the post-translational modification of proteins and do not always result in proteins that undergo proper folding (Carlson et al., 2012). As a result, it is possible that some proteins here did not fold into the correct confirmation, resulting in lower immunoreactivity values due to lack of functional epitopes.

The transferrin-binding protein TbpA was included in this analyses but it was truncated into two products (MhL044A_01700_465A, MhL044A_01700_465B) ranking 84$^{th}$ and 105$^{th}$, respectively. It is possible that the full length protein may have produced a higher immunoreactivity score if truncation disrupted its natural epitope. A similar truncation was performed on the TbpB gene. The N-terminal portion of the protein (MhL044A_01701_292A) ranked 186th while the C-terminal portion (MhL044A_01701_293B) ranked 25$^{th}$.

A limitation of the approach used here is that candidate selection was designed to target proteins with high sequence similarity within a large portion of the bacterial population. Those genes that express sequence variation less than 95% identity over 90% length would not have clustered together and as a result would not have had been represented in over 50% of the dataset and included for further bioinformatic analysis. It is likely that this was the case with the GS60 antigen which was not selected in the initial screening.

The immunoreactivity of proteins to serum provides a quantitative measure for humoral responses indicating the magnitude of antibody generation in response to challenge. The ability for an antigen to elicit antibody production does not necessarily correlate with the degree of protection from infection. It is possible that candidates not readily displayed on the cell surface and thus unavailable to the immune system for opsonization may still be taken up and displayed by antigen-presenting cells resulting in antibody generation. Further investigation into the quality of antibody response, by examining affinity, avidity, diversity and capacity to neutralize, needs to be undertaken for the

candidates here before they can be reasonably evaluated for use in vaccine formulation. An investigation into the cellular immune responses of each candidate is also required as this arm of the immune system is intimately linked with humoral immunity, the activities of T helper cells required to promote a robust and enduring antibody response to infection.

**6.5 Conclusion**

In conclusion, a RV approach was used to identify potential vaccine candidates in *M. haemolytica* and a high throughput method employed to evaluate their immunoreactivity. The method as presented was not optimized to maximize the success of translated products beyond the kit requirements. Even so, over 80% of the protein candidates were expressed and bound effectively to the $Ni^{+2}$ plates. However, this system could potentially be adjusted further to improve the expression, stability and binding affinity of difficult candidates by using protein structure prediction to identify and truncate hydrophobic regions that could lead to protein instability or inaccessibility of the C-terminal Hisx6 tag for binding. The assay identified multiple vaccine candidates of which Ssa1 was ranked the highest; this is a serine protease that others have identified as being highly immunogenic. An additional four high ranking proteins, the competence protein ComE, the solute binding protein, and a molybdenum ABC transporter which have not been previously examined in serological assays were also shown to hold promise for vaccine design against this species of respiratory pathogen. The development of efficacious vaccines against agents of respiratory disease in cattle will help reduce the negative economic impact BRD has on the North American industry. The framework generated from this research can be used for vaccine development against other pathogens of this complex respiratory infection in cattle.

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## 7 General discussion, conclusions and future directions

### 7.1 General discussion

Although bovine respiratory disease research originated in the 1800's (Taylor et al., 2010) this condition is still one of the foremost health problems faced by beef producers in North America. A complicating factor towards resolving this disease is its complex etiology. A component of this project examined the viral and bacterial agents contributing to BRD mortalities in North America. Studies with similar intent have occurred in the past 10 years (Booker et al., 2008; Fulton, 2009; Gagea et al., 2006) but these did not incorporate BRD cases encompassing as broad a geographic range and did not characterize bacterial isolates as extensively as this study. I found *Mannheimia haemolytica* to be the most frequently recovered bacterial pathogen from BRD cases. However, all other agents with the exception of bovine herpesvirus 1 were also detected in BRD mortalities including *P. multocida, H. somni,* M. *bovis*, bovine viral diarrhoea virus, bovine parainfluenza 3 virus, and bovine respiratory syncytial virus. The lack of bovine herpesvirus 1 was not unexpected as this virus can be difficult to detect post-infection (Pastoret and Thiry, 1985).

A key finding of this study was the prevalence of integrative conjugative elements (ICEs) detected in isolates of *M. haemolytica, P. multocida* and *H. somni*. Some of these ICEs containing gene cassettes conferred resistance to all antimicrobials used in BRD therapy, with the exception of ceftiofur. Although a similar multidrug resistant ICE profile was identified previously in *P. multocida* (Michael et al., 2012), the work here was the first to report these elements in *M. haemolytica* and *H. somni* from beef calves.

Multidrug resistance conferring ICEs (i.e. resistance to greater than six antibiotic classes) were only identified in isolates collected from American feedlots, occurring in over 80% of *M. haemolytica*

collected from Nebraska and Texas. The average feedlot in the USA contains approximately four times the number of cattle compared to those in Canada (Galyean et al., 2011). And with the five largest feeding operations controlling 20% of feeding capacity in the USA (Galyean et al., 2011), the intensive aggregation of animals in confined spaces is likely translating into more frequent drugs use to prevent disease. The congregation of large numbers of cattle within feedlots can also be predicted to facilitate the ease by which pathogenic strains of bacteria can spread within herds. There is also the possibility that *Pasteurellaceae* species carrying AMR are originating from other agricultural sectors in this region, like swine or poultry, where *P. multocida* and *M. haemolytica* also cause disease and are targets for antimicrobial therapies (Angen et al., 1999; Ewers et al., 2006; Kehrenberg et al., 2001). Further investigation into the dispersal of ICEs within North America will likely shed some light on their origin and give insight into management practices that could be promoting their development and spread.

The detection of multidrug resistant *M. haemolytica, P. multocida* and *H. somni* together from BRD mortalities is significant. Currently up to 50% of animals receive antibiotics either prophylactically or therapeutically to prevent or treat this disease (Checkley et al., 2010; Hilton, 2014). Thus, the failure of therapies used in feedlots to control BRD will likely result in a substantial increase in death loss. Given the current dependency of antibiotics, without working therapeutics it is unlikely that the beef industry would be able to maintain its current structure, as losses to disease would undercut profitability.

Alternatives to antibiotic use for BRD management exist and include preconditioning programs that promote animal immune health by reducing stress and pathogen exposure through weaning, vaccination, dehorning, deworming and treatment for external parasites prior to the animal moving to the feedlot (Schumacher et al., 2012). Animal husbandry practices are also used and include increasing the forage level in the diet, reducing the number of animals in pens, minimizing the mixing of cattle from multiple sources and ensuring adequate ventilation (Edwards, 2010). The use of ancillary anti-inflammatory drugs has also been promoted as a means to decrease the severity of clinical symptoms and inflammation induced lung damage, reduce fever and increase appetite, but there is little or no clinical evidence to support the reliability or effectiveness of these treatments (Francoz et al., 2012).

Concern exists over the potential transfer of multidrug resistant ICEs into zoonotic pathogens. This project examined the mobility of ICEs between *Pasteurellaceae* species and *E. coli* showing that these elements could conjugate *in vitro* between species. Further investigation will be required to determine the likelihood of such a transfer event in the feedlot setting, but the work here shows the possibility exists and that it would be prudent to continue surveillance of these important mobile

genetic elements in the agricultural sector. Furthermore, surveillance of ICE containing bacterial pathogens in feedlots will likely play an increasingly important role in providing veterinarians the information they need to develop drug administration regimes to effectively combat multidrug resistant infections.

Prior to initiation of this study whole genome sequence data was only available for three strains of *M. haemolytica*: a serovar 1 strain isolated from a beef calf, one serovar 2 isolated from a cattle and one serovar 2 isolated from sheep (Gioia et al., 2006; Lawrence et al., 2010). Subsequent sequencing projects were submitted to NCBI in the interim, those *M. haemolytica* genomes available at the time Chapters 5 and 6 were performed were included in the analysis as specified in those studies. From this project, draft genomes of 11 *M. haemolytica* were generated representing serovars S1, S2 and S6. This included the first documented whole genome sequence of a serotype 6 strain of *M. haemolytica*. As anticipated, sequence diversity was the highest in S2 strains, with S1 and S6 exhibiting similar genomic characteristics in terms of the presence of bacteriophage and spacer arrays within CRISPR-Cas. Comparative analysis highlighted two spacers within S1 and S6 CRISPR arrays with the potential to contribute to *M. haemolytica* virulence through the regulation of expression of sialic acid. These spacers were lacking in S2 strains supporting our understanding of S1 and S6 playing a more central role in bovine disease. Although rare, evidence of CRISPR-Cas mediated regulation of virulence factors has been previously described (Sampson and Weiss, 2013). It would be interesting to determine if a similar mechanism was working in CRISPR-Cas from other respiratory pathogens including *P. multocida* and *H. somni*. A comparative analysis of CRISPR arrays from bacterial species infecting the respiratory tract of cattle, focusing on self-targeting spacers could highlight factors that allow these microbes to avoid host immune responses and persist in this niche.

Pan-genome analysis was performed with the 11 *M. haemolytica* genomes generated here with an additional 15 *M. haemolytica* genomes present in public databases. All of the coding sequences in the dataset were identified and further screened using bioinformatics tools for signatures that identify their gene products as being cell surface associated. To my knowledge, this is the first application of reverse vaccinology (RV) to compare pathogenic and non-pathogenic strains of *M. haemolytica* to identify potential vaccine candidates.

Although RV provides a means to rapidly identify vaccine candidates, evaluation of those proteins for their suitability in vaccine formulation still presents a challenge. This project utilized the unique application of a cell-free translation system coupled with ELISA to screen proteins for their immunoreactivity. By using this high throughput strategy, within a manner of weeks 291 protein

candidates were screened using serum generated in calves challenged with S1, S2 or S6 strains of *M. haemolytica*. This is a massive reduction in time from the months expected to be required for the traditional approach of cloning, expressing and purifying each protein.

Of the top five candidates identified here, two were periplasmic components of the ABC transport system. It is unclear with serology testing if the antibodies generated against these proteins result in protection. As periplasmic components, it is unlikely that they are targets for antibody opsonization. However, antibodies generated against the lipoproteins of similar ABC systems have been shown to provide protection, the theory being that the antibodies diffuse through the cell wall and interfere with the biological function of the transport system (Garmory and Titball, 2004). The challenge trial used to generate the sera for serology screening required the heavy dosing of animals with bacterial inoculant. It is possible that degraded cell components as part of this mixture were taken up, displayed by antigen presenting cells and elicited a host antibody response in a manner not reflective of natural *M. haemolytica* infection. This is a limitation of experimental infection and although not for certain, it is possible that sera obtained from naturally infected animals could be less immunoreactive to these periplasmic components.

It is important to note that RV analysis is limited to proteins and cannot be used to identify non-protein coding antigens like lipopolysaccharides or lipids. Also, correlates for protection are rare making screening for protective immunity the rate limiting step for this application (Bertholet et al., 2014). Other limitations to this work include the scope of the dataset used. Although we targeted samples from a wide geographic range, pan-genome analysis revealed that we could add hundreds more genomes to the analysis and not fully cover the diversity within *M. haemolytica* populations. In addition, all bioinformatic screening is based on cut-off values which are user defined. We applied cut-offs with the intent to target antigens that would be protective across at least half of the strains analyzed. Alternative screening parameters couldl result in the identification of different antigen targets.

This project identified multiple candidates for use in vaccine design against *M. haemolytica,* but did not fully evaluate their suitability for use in vaccine formulation. Serological assays only examine the likelihood for antigens to bind to antibodies, and do not necessariy reflect protective efficacy. As a result animal models are still required to measure *in vitro* efficacy of the antigens identified using *in silico* tools (Bambini and Rappuoli, 2009). Ideally a comprehensive immune stimulus profile comprised of information about immunoreactivity, cellular immune response and *in vivo* protein expression should be used to determine the suitability of each vaccine candidate prior to use in animal challenge trials.

**7.2 Future work**

Future work should examine cell-mediated immune responses. Epitope prediction programs can be employed to generate a short list of antigen candidates from the original pan-genome analysis described in Chapter 6. Advancements in software have allowed for reasonable prediction of class I MHC peptide binding but class II MHC peptide binding prediction programs are less reliable and still need further improvements (Sette and Rappuoli, 2010). However, as functional and structural genomics progress and bioinformatic resources grow, epitope prediction will also likely improve making vaccine design more targeted and streamlined.

In addition to cell-mediated immune assays *in vivo* protein expression data is required to determine if proteins are produced in sufficient abundance to act as targets for the host immune system. Although RV can be used to identify genes it cannot determine if the genes are expressed during infection. This information can only be obtained through proteomic or transcriptomic analysis, the latter possible with RNAseq studies of lung lavage samples collected from animals experimentally infected with *M. haemolytica* (Reddy et al., 2012).

Once good antigen candidates are identified, fusion proteins or chimeras can be engineered to facilitate patents and reduce the cost of protein expression that can be a detriment to vaccine production (Lakshmi et al., 2013). Vaccine delivery is also a component of this work that can be explored further. The use of vesicles as delivery systems is an area of current focus (Watson et al., 2012) as well as the use of DNA based vaccines (Yang et al., 2015).

Another logical progression from this project is to apply the approach used to other pathogens of BRD. Comparative analysis of *M. haemolytica*, *H. somni* and *P. multocida* with representative commensals can be used to highlight targets that may be protective against all three organisms. Difficulties in this strategy may be to identify targets that are not cross reactive to other non-target species. It is important to note that although RV can deliver antigen candidates in a relatively short period of time, the identification of a suitable antigen is one step in vaccine design and full vaccine development requires formulation, proof of principal, clinical trials, patenting, licencing and approval, and marketing.

Lastly, multiple avenues exist here to further characterize the ICEs discovered. Sequence analysis of the *P. multocida* and *H. somni* isolates could be performed to resolve these elements as they were only examined using phenotypic analysis and PCR. Further characterisation of ICE components focusing on the type 4 secretion systems will also provide more insight into the mechanisms of transfer and maintenance of these MGE. Larger scale surveillance of ICEs in North America should be

undertaken in an attempt to identify the source and relatedness of these elements within animal production systems. This could include metagenomic screening of animal and environmental samples and possibly those from humans. The threat of movement of ICEs into zoonotic pathogens should also be considered and evaluated. Conjugation studies between ICE and pathogens related to human health should be pursued.

Bovine respiratory disease is a complex, multifactorial condition. As a result any solution to manage it will likely also be complex and multifaceted. It is tempting to look at antimicrobials as the silver bullet for disease in feedlot settings, and to some degree the applications of these therapies are very effective and necessary. However, sustainability of this approach to controlling infection is always a challenge. At this stage the application of antibiotics is driven by ground level policy, based on the priorities of the individual producers. A more global view of the relationships between antibiotic applications and the consequences for resistance development is necessary to help develop a framework for decision making around prudent usage of these medicines in agriculture.

With the advancements in omics technologies vaccine development has undergone some significant changes and the potential exists to address some of the diseases left unchallenged by conventional methodologies. Vaccines are a better strategy for prevention of disease than antimicrobials as they are more efficient, cost effective and sustainable. However, even with the use of vaccines and antimicrobials it is not likely that BRD will be completely eradicated from production systems. The majority of BRD agents are commensals found in healthy animals. There is the possibility that effective vaccination that removes the threat of one BRD agent will see it replaced by another. To some extent this may be occurring already as there has been a shift in respiratory profiles from acute *M. haemolytica* associated disease to more chronic *H. somni* and *M. bovis* associated infection (Booker et al., 2008; Gagea et al., 2006). The solution to BRD likely lies in a new paradigm for beef production. However, current focus on disease prevention through encouraging preconditioning programs at the cow-calf level and through the development and use of effective vaccines are likely going to become key initiatives for BRD management in the future.

**7.3 Concluding remarks**

By developing a successful vaccine providing broad protection against pathogenic *M. haemolytica*, the Alberta and Canadian beef sector will be one step closer to effectively controlling BRD. It is well recognized that *M. haemolytica* is a major component of BRD. However, this project has generated a foundation to identify vaccine candidates against other etiological agents associated with

BRD using RV. Ultimately, if feedlots were able to manage BRD using vaccination it would dramatically impact the feedlot stage of the beef industry value chain and reduce the use of antimicrobials.

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## Appendix A

Table A1 *Mannheimia haemolytica* from public databases used in pan-genome analysis

| Isolate | | Accession No. | Serotype |
|---|---|---|---|
| *M. haemolytica* | D153 | NC_021743.1 | 1 |
| *M. haemolytica* | MhBrain2012 | ATSZ00000000.1 | 1 |
| *M. haemolytica* | D193 | ATSY00000000.1 | 1 |
| *M. haemolytica* | PHL213 | NZ_AASA00000000.1 | 1 |
| *M. haemolytica* | D171 | NC_021738.1 | 2 |
| *M. haemolytica* | D35 | AUNK00000000.1 | 2 |
| *M. haemolytica* | Bovine A2 | NZ_ACZY00000000.1 | 2 |
| *M. haemolytica* | D174 | NC_021739.1 | 6 |
| *M. haemolytica* | D38 | AUNL00000000.1 | 6 |
| *M. haemolytica* | USDA-ARS-USMARC-185 | NC_020834.1 | 6 |

Table A2 Percent identities of Cas genes, virulence factors, and ICE associated genes from 11 *Mannheimia haemolytica* isolates

| | | | | | % pairwise identity (% identical sites) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Reference ID[a] | AA length | % pairwise identity (% identical sites) | sequence length | all | S1/S6 | S1 | S6 | S2 |
| CRISPR-Cas | | | | | | | | | |
| Cas1 | n/a | 337 | 98.8 (97.0) | 1014 | 97.8 (94.9) | 100 (99.9) | 100 (100) | 100(99.9) | 99.9 (99.9) |
| Cas2 | n/a | 97 | 100 (100) | 294 | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| Cas2b | n/a | 90 | 100 (100) | 273 | | | | | |
| Cas2 vs Cas2b | n/a | 99 | 77.9 (24.2) | 299 | 85.3 (49.5) | | | | |
| Cas3 | n/a | 762 | 99.5 (98.2) | 2289 | 99.2 (97.1) | 100 (100) | 100 (100) | 100 (100) | 98.3 (97.4) |
| Cas4 | n/a | 224 | 95.6 (89.7) | 675 | 95.0 (88.6) | 99.9 (99.7) | 99.9 (99.9) | 99.9 (99.9) | 100 (100) |
| Cas5 | n/a | 225 | 99.8 (99.1) | 678 | 99.5 (97.6) | 100 (100) | 100 (100) | 100 (100) | 98.4 (97.6) |
| Cas7 | n/a | 287 | 99.6 (99.0) | 864 | 99.4 (97.9) | 100 (100) | 100 (100) | 100 (100) | 99.2 (98.8) |
| Cas8 | n/a | 594 | 99.7 (98.5) | 1785 | 98.6 (95.7) | 99.8 (99.3) | 100 (100) | 99.6 (99.3) | 98.9 (98.4) |
| LPS Pathway | | | | | | | | | |
| 3-deoxy-D-manno-octulosonic-acid transferase WaaA | MHA_0068 | 427 | 99.9 (99.8) | 1281 | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| possible glycosyltransferase WcaA-like | MHA_0100 | 267 | 99.3 (99.8) | 801 | 99.9 (99.6) | 100 (100) | 100 (100) | 100 (100) | 99.8 (99.8) |
| possible glycosyltransferase WcaA-like | MHA_0101 | 323 | 99.7 (99.1) | 966 | 99.8 (99.3) | 100 (100) | 100 (100) | 100 (100) | 99.6 (99.4) |
| Pasteurellaceae conserved hypothetical protein | MHA_0102 | 395 | 99.9 (99.5) | 1188 | 100.0 (99.8) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.8) |
| possible glycosyltransferase | MHA_0103 | 261 | 99.9 (99.6) | 786 | 99.9 (99.6) | 100 (100) | 100 (100) | 100 (100) | 99.7 (99.6) |
| possible O-antigen | MHA_0105 | 401 | 99.5 (98.3) | 1207 | 99.9 | 100 | 100 | 100 | 99.9 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| export protein | | | | | (99.6) | (99.9) | (99.9) | (100) | (99.8) |
| UDP-N-acetylglucosamine 2-epimerase WecB | MHA_0106 | 394 | 99.7 (99.0) | 1185 | 99.9 (99.5) | 100 (100) | 100 (100) | 100 (100) | 99.7 (99.6) |
| heptosyltransferase II (inner core) WaaF | MHA_0191 | 345 | 99.7 (99.9) | 1038 | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| phosphomannomutase ManB | MHA_0517 | 550 | 99.7 (99.1) | 1653 | 99.5 (97.9) | 100 (100) | 100 (100) | 100 (100) | 98.8 (98.2) |
| UDP-N-acetylglucosamine 2-epimerase WecB | MHA_0521 | 371 | 99.1 (98.4) | 1116 | 98.2 (96.7)[8] | 98.2 (96.7)[8] | 100 (100)[4] | 98.3 (96.7)[4] | nd |
| ECA biosynthesis protein WecC | MHA_0522 | 423 | 97.4 (95.5)[8] | 1207 (1272) | 96.4 (93.8)[8] | 96.4 (93.8)[8] | 100 (100)[4] | 98.3 (96.5)[4] | nd |
| UDP-N-acetyl muramyl pentapeptide phosphotransferase WecA | MHA_0726 | 354 | 100 (100) | 1065 | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| lipopolysaccharide chain length determinant protein WzzB | MHA_0727 | 271 | 99.9 (99.6) | 813 | 100 (99.9) | 100 (99.9) | 99.9 (99.9) | 100 (100) | 100 (100) |
| UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase WecC | MHA_0728 | 414 | 99.9 (99.8) | 1245 | 99.9 (99.7) | 100 (99.8) | 100 (100) | 100 (99.9) | 99.8 (99.8) |
| probable fucosamine acetyl transferase | MHA_0729 | 214 | 99.9 (99.5) | 645 | 99.2 (95.7) | 100 (100) | 100 (100) | 100 (100) | 97.1 (95.7) |
| ECA biosynthesis protein WecE | MHA_0730 | 473 | 100 (100) | 1422 | 99.9 (99.7) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.8) |
| lipopolysaccharide N-acetylglucosaminyltransferase | MHA_0825 | 398 | 100 (100) | 1197 | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| ADP-glyceromanno-heptose 6-epimerase HldD | MHA_1050 | 308 | 100 (100) | 927 | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.9) |
| UDP-glucose 4-epimerase GalE | MHA_1349 | 338 | 99.8 (99.4) | 1017 | 99.9 (99.8) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.8) |
| lipid A acyltransferase | MHA_1392 | 316 | 100 (100) | 951 | 99.9 (99.8) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.9) |
| glycosyltransferase LpsA | MHA_1558 | 263 | 99.8 (99.6) | 792 | 99.9 (99.7)[9] | 99.9 (99.7)[6] | 100 (100)[2] | 99.9 (99.7)[4] | 100 (100)[3] |
| lipid A acyltransferase | MHA_1562 | 313 | 99.9 (99.7) | 942 | 99.9 (99.8) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| heptosyltransferase II (inner core) WaaF | MHA_1600 | 343 | 99.8 (99.1) | 1032 | 99.9 (99.5) | 100 (99.9) | 100 (100) | 99.9 (99.9) | 99.8 (99.7) |
| O-antigen ligase WaaL | MHA_1845 | 418 | 100 (100)[8] | 1257 | 100 (100)[8] | 100 (100)[8] | 100 (100)[4] | 100 (100)[4] | nd |
| dTDP-glucose 4,6-dehydratase RmlB | MHA_1846 | 335 | 100 (100)[8] | 1008 | 100 (100)[8] | 100 (100)[8] | 100 (100)[4] | 100 (100)[4] | nd |
| conserved hypothetical protein | MHA_1847 | 193 | 100 (100)[8] | 582 | 100 (100)[8] | 100 (100)[8] | 100 (100)[4] | 100 (100)[4] | nd |
| possible LPS sugar transferase | MHA_1848 | 423 | 100 (100)[8] | 1272 | 100 (100)[8] | 100 (100)[8] | 100 (100)[4] | 100 (100)[4] | nd |
| LPS chain length determining protein Wzz | MHA_1853 | 375 | 100 (100)[6] | 1128 | 100 (100)[6] | 100 (100)[6] | 100 (100)[2] | 100 (100)[4] | - |
| phosphomannomutase ManB | MHA_2240 | 444 | 99.9 (99.5) | 1335 | 99.9 (99.6) | 100 (99.9) | 100 (99.9) | 100 (100) | 99.8 (99.7) |
| D,D-heptose 1-phosphate adenosyltransferase/7-phosphate kinase HldE | MHA_2564 | 475 | 99.7 (99.4) | 1428 | 99.9 (99.7) | 100 (100) | 100 (100) | 100 (100) | 100 (99.9) |
| heptosyltransferase II (inner core) WaaF | MHA_2704 | 345 | 99.4 (98.3) | 1038 | 99.8 (99.3) | 100 (100) | 100 (100) | 100 (100) | 99.6 (99.3) |
| heptosyltransferase II (inner core) WaaC | MHA_2705 | 319 | 99.9 (99.7) | 960 | 99.9 (99.7) | 100 (99.9) | 99.9 (99.9) | 100 (100) | 99.9 (99.8) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 3-deoxy-8-phosphooctulonate synthase | MHA_1912 | 284 | 99.7 (98.9) | 855 | 98.6 (95.8) | 100 (100) | 100 (100) | 100 (100) | 97.2 (95.8) |
| 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase | MHA_2646 | 179 | 99.8 (99.4) | 540 | 99.9 (99.8) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.8) |
| 3-deoxy-D-manno-octulosonate cytidylyltransferase | MHA_0212 | 251 | 100 (100) | 756 | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| 3-deoxy-D-manno-octulosonic-acid transferase | MHA_0068 | 426 | 99.9 (99.8) | 1281 | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase | MHA_0687 | 264 | 100 (100) | 795 | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.9) |
| ADP-L-glycero-D-manno-heptose-6-epimerase | MHA_1050 | 308 | 100 (100) | 927 | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.9) |
| D,D-heptose 1,7-bisphosphate phosphatase | MHA_0543 | 182 | 99.3 (98.4) | 549 | 99.4 (98.4) | 100 (100) | 100 (100) | 100 (100) | 99.5 (99.3) |
| lipid-A-disaccharide kinase | MHA_1351 | 333 | 99.8 (99.4) | 1002 | 99.7 (99.0) | 100 (100) | 100 (100) | 100 (100) | 99.4 (99.1) |
| lipid-A-disaccharide synthase | MHA_0736 | 392 | 99.8 (99.5) | 1179 | 99.9 (99.8) | 100 (100) | 100 (100) | 100 (100) | 99.9 (99.9) |
| Lipopolysaccharide kinase - 3-deoxy-D-manno-octulosonic acid kinase (Kdo/WaaP) | MHA_0049 | 235 | 99.1 (97.9) | 708 | 98.9 (97.5) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| phosphoheptose isomerase 583 | MHA_1457 | 193 | 100 (100)[10] | 582 | 99.9 (99.7)[10] | 100 (99.8)[8] | 99.9 (99.8)[4] | 100 (100)[4] | 100 (100)[2] |
| phosphoheptose isomerase 585 | MHA_2736 | 194 | 99.8 (99.5) | 585 | 99.9 (99.7) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| rfaE - D-alpha,beta-D-heptose 7-phosphate 1-kinase | MHA_2564 | 475 | 99.7 (99.4) | 1428 | 99.9 (99.7) | 100 (100) | 100 (100) | 100 (100) | 100 (99.9) |
| UDP-2,3-diacylglucosamine hydrolase | MHA_1959 | 233 | 100 (100) | 702 | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase | MHA_0689 | 341 | 100 (100) | 1026 | 99.6 (98.6) | 100 (100) | 100 (100) | 100 (100) | 99.1 (98.6) |
| UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase | MHA_2062 | 306 | 99.9 (99.7) | 921 | 99.9 (99.7) | 100 (99.9) | 100 (100) | 99.9 (99.9) | 100 (100) |
| Virulence factors neuraminidase (sialidase) | MHA_1532 | 791 | 99.8 (98.7) | 2377 | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| gcp gene - O-sialoglycoprotein endopeptidase | MHA_1559 | 343 | 100 (100) | 1032 | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| filamentous hemagglutinin (FhaB) | MHA_0866 | 3214 | 99.6 (99.2) | 9465-9645 | 99.5 (99.0)[7] | 100 (100)[5] | 100 (100)[4] | - | 100 (100)[2] |
| hemolysin activation/secretion protein FhaC | MHA_0867 | 598 | 98.8 (96.3) | 1797 | 98.7 (96.3) | 99.8 (99.4) | 100 (99.9) | 99.7 (99.4) | 99.2 (98.8) |
| Ssa1 | MHA_2492 | 942 | 93.3 (81.7) | 2253-2829 | 94.0 (83.1) | 99.9 (99.4) | 100 (100) | 99.7 (99.4) | 96.0 (94.6) |
| PlpE | MHA_1514 | 360 | 84.6 (60.6) | 1068-1071 | 88.7 (71.1)[9] | 100 (100)[7] | 100 (100) | 100 (100)[3] | 100 (100)[2] |
| autotransporter adhesins | MHA_2701 | 2023 | 100 (99.9) | 5658-6072 | 100 (99.9)[8] | 100 (99.9) | 100 (100) | 100 (100) | nd |
| autotransporter | MHA_1367 | 1383 | 100 (99.9) | 4152 | 100 | 100 | 100 | 100 | nd |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| adhesins | | | | | (99.9)[8] | (99.9) | (100) | (100) | |
| autotransporters | MHA_0563 | 1398 | 100 (99.9) | 4197 | 100 (99.9)[10] | 100 (100)[7] | 100 (100) | 100 (100)[3] | 100 (100) |
| autotransporters | MHA_2800 | 1503 | 99.9 (99.4) | 4512 | 99.9 (99.8)[9] | 100 (100) | 100 (100) | 100 (100) | - |
| HmbR1 - hemoglobin receptor | MHA_1639 | 614 | 99.9 (99.8) | 1767-1845 | 99.9 (99.8) | 100 (99.9) | 100 (100) | 100 (100) | 100 (100) |
| HmbR2 - hemoglobin receptor | MHA_2261 | 719 | 99.9 (99.4) | 2160 | 100 (99.9) | 100 (99.9) | 100 (99.9) | 100 (100) | 100 (100) |
| HxuA - hemophore | MHA_1004 | 940 | 98.1 (94.3) | 2817-2823 | 97.9 (94.0) | 100 (100) | 100 (100) | 100 (100) | 98.2 (97.3) |
| TbpA - transferrin-binding protein | MHA_0196 | 937 | 95.8 (83.8) | 2787-2811 | 95.1 (78.5) | 100 (99.9) | 100 (99.9) | 100 (100) | 86.5 (79.8) |
| TbpB - transferrin-binding protein | MHA_0197 | 605 | 82.9 (52.4) | 1752-1767 | 85.0 (57.1) | 99.7 (99.1) | 99.8 (99.5) | 99.8 (99.5) | 75.8 (63.9) |
| FhuE like - ferric hydroximate siderophore receptor | MHA_2388 | 710 | 99.6 (99.2) | 1704-2133 | 99.7 (99.3)[10] | 100 (100) | 100 (100) | 100 (100) | 100 (100)[2] |
| ferric hydroximate siderophore receptor | MHA_1541 | 704 | 99.1 (96.7) | 1917-2076 | 99.8 (99.3) | 99.9 (99.7) | 100 (99.9) | 100 (100) | 99.7 (99.6) |
| Fur - ferric uptake regulator | MHA_2790 | 146 | 99.9 (99.3) | 441 | 100 (99.8) | 100 (100) | 100 (100) | 100 (100) | 99.8 (99.8) |
| GS60 - outer membrane lipoprotein | MHA_2734 | 573 | 100 (100) | 1772 | 99.9 (99.8) | 100 (99.9) | 100 (99.9) | 100 (100) | 100 (100) |
| OmpA | MHA_1054 | 378 | 97.7 (93.9) | 1119-1137 | 98.4 (95.9)[10] | 100 (100)[7] | 100 (100) | 100 (100) | 98.0 (97.0) |
| LuxS | MHA_2788 | 169 | 99.2 (97.6) | 510 | 98.1 (94.1) | 100 (100) | 100 (100) | 100 (100) | 96.1 (94.1) |
| LktC | MHA_0253 | 184 | 93.3 (79.3) | 456-555 | 97.8 (92.4) | 99.9 (99.8) | 99.9 (99.8) | 100 (100) | 98.5 (97.8) |
| LktA | MHA_0254 | 954 | 94.9 (86.3) | 2862 | 93.7 (82.9) | 100 (100) | 100 (100) | 100 (100) | 95.3 (93.0) |
| LktB | MHA_0255 | 708 | 99.8 (99.2) | 2127 | 98.5 (95.7) | 100 (100) | 100 (100) | 100 (100) | 98.7 (98.0) |
| LktD | MHA_0256 | 478 | 99.9 (99.8) | 1437 | 100(99.9 ) | 100 (99.9) | 100 (100) | 100 (99.9) | 100 (100) |
| ICE associated genes | | | | | | | | | |
| TraC | n/a | 945 | 98.4 (96.7) | 2838 | 95.1 (89.7) | 96.0 (89.7) | 93.5 (89.7) | 100 (100) | - |
| TraD | n/a | 734 | 98.6 (97.3) | 2202 | 95.5 (91.0) | 96.1 (91.0) | 94.0 (91.0) | 100 (100) | - |
| TraG | n/a | 490-506 | 82.6 (38.7) | 1473-1519 | 93.4 (84.2) | 95.4 (84.2) | 91.4 (84.1) | 99.9 (99.9) | - |
| TraU | n/a | 313 | 97.4 (93.3) | 942 | 96.0 (89.6) | 100 (100) | 94.8 (89.6) | 100 (100) | - |
| TrbI | n/a | 484 | 94.6 (89.3) | 1455 | 92.9 (85.8) | 93.9 (85.8) | 90.6 (85.8) | 100 (100) | - |
| tyrosine recombinase | n/a | 254 | 99.1 (96.1) | 765 | 98.1 (92.9) | 98.4 (93.5) | 96.7 (93.5) | 100 (100) | - |
| tyrosine recombinase 900bp | n/a | 299 | 100 (100) | 900 | 100 (100) | - | - | - | - |
| Relaxase/ TraI | n/a | 516-661 | 96.0 (89.9) | 1551-1986 | 96.1 (89.5) | 97.4 (89.5) | 95.0 (89.6) | 100 (100) | - |
| multicopper oxidase | n/a | 515 | 100 (100) | 1548 | - | - | - | - | - |
| DNA topoisomerase | n/a | 683 | 96.1 (92.2) | 2052 | 92.4 (84.7) | 93.5 (84.7) | 89.8 (84.7) | 100 (100) | - |
| ParB | n/a | 548-549 | 95.8 (91.6) | 1647-1650 | 94.0 (88.1) | 94.9 (88.1) | 92.0 (88.1) | 100 (100) | - |
| pilL | n/a | 209-210 | 94.3 (88.6) | 630-633 | 94.5 (89.0) | 95.3 (89.0) | 92.6 (89.0) | 100 (100) | - |

[a]reference locus ID given from M. haemolytica sv. 1 PHL213; accession NZ_AASA00000000.1. n/a, not provided

Table A3 The gene targets, primers used for expression, and ranking based on immunoreactivity screening using sera raised in cattle experimentally infected with strains of either serotype 1, serotype 2 or serotype 6 of *Mannheimia haemolytica*

| Gene target locus ID[a] | Forward Primer/ reverse primer (5'-3')[b] | Protein length (aa) | PSORT Location | Rank for immuno-reactivity against anti-S1 sera[c] | Rank for immuno-reactivity against anti-S2 sera[c] | Rank for immuno-reactivity against anti-S6 sera[c] | Rank for overall average immuno-reactivity |
|---|---|---|---|---|---|---|---|
| MhH23_00891 | CAAAATACTGCTCAAA ACAGTG/ ATTATTCCTCAATGACC TCGG | 210 | Unknown | 122 | 103 | - | 139 |
| MhH23_00892 | ACTACATCAATAGAAA GTAGCAC/ ATAAACGAGAGATTGC CATTCAC | 245 | Unknown | 129 | 121 | - | 152 |
| MhH23_00894 | TTAACGGTAATCGCTG ATTTCG/ GGTTTCAGTAATCAGG GC | 168 | Unknown | 23 | 6 | - | 16 |
| MhH23_00901 | GCATCCAAAATCCCAG ACTTC/ TCCGACTGCAAGTTTG ACTAAC | 129 | Cytoplasmic | - | - | - | - |
| MhL024A_00046 | GCAACAGAACCTACAA AAAC/ GAATCTTTGCTGTAATT CTACG | 194 | Periplasmic | - | - | - | - |
| MhL024A_00488 | CAAAGCATCAAGACGG AGATG/ TTTGTATTTTTTGTGAT ATTCACG | 123 | Unknown | - | - | - | - |
| MhL024A_00497_357A | GACGGTTATTTAAGTA ACGA/ AACAAATGTATAAAGA GCTTC | 357 | Unknown | 52 | 54 | 73 | 69 |
| MhL024A_00497_358B | CGTGCAATGGGGTTAT TTAC/ TTGATAGTGATAATTT GCGTGT | 358 | Unknown | 82 | 77 | 104 | 106 |
| MhL024A_01035_562A | GAATTGCCAACCCAAC AG/ AGGATTTTCAAATGAA ATATAAGTGA | 562 | Cytoplasmic | 96 | 99 | 111 | 123 |
| MhL024A_01035_563B | GTCTGGACTTGGGGAT TAAAA/ TCGTTCAAACTCAAAG CGT | 563 | Cytoplasmic | 8 | 9 | 8 | 10 |
| MhL024A_01315 | AATGATCCTTTCTTTGG AG/ TAATTTGATGTTAATTT CATAAGGG | 137 | Unknown | 124 | 123 | 181 | 178 |
| MhL024A_01509_334A | TCAATCACTGATAAACT GGAA/ GACAGGTACACTACTT GAGC | 334 | Unknown | 91 | 93 | 124 | 125 |
| MhL024A_01509_334B | ACTCGTGGCTTGATTG AAA/ ATTTTTACCAAGAATAT AATCAGCT | 334 | Unknown | 24 | 34 | 30 | 31 |
| MhL024A_01510 | AACGTAATATTTTCCTG TACCAGC/ AGTATTCATCAAATGA ACATCAAAC | 142 | Unknown | 121 | 135 | 172 | 177 |

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|---|---|---|---|---|---|---|---|
| MhL024A_01515 | GATAGTTGAAGTTTTTAGACAC/TTGTTTTTGGCGACTACTATAA | 221 | Unknown | - | - | - | - |
| MhL024A_01796 | AAGCATAAATTCAAACTAAACC/CTCTGTTTTATATTTTTCAATGTAAG | 356 | Periplasmic | - | - | - | - |
| MhL024A_01813 | ATGACAGAAAAAGCTGAACAAG/CTTGGTTGCAGAAATGGATTTTG | 115 | Unknown | 120 | 127 | 142 | 162 |
| MhL024A_02718 | ATGATGAGAAAATTAGCAAAACAG/AATTGGAGCAGAGGTAGGT | 220 | Extracellular | 102 | 108 | 138 | 141 |
| MhL044A_00003 | CAACCGACCTTAACCGTTTAC/CCAGCTCTTTGACCAATCGG | 317 | Periplasmic | - | - | - | - |
| MhL044A_00027 | GGTAAGTCTATTCATATTAACTCGG/TACATTTTTTAAGAAAATTTGGCG | 298 | Periplasmic | 79 | 130 | 117 | 132 |
| MhL044A_00028 | TTCCACGGATATGCACGTTC/AGCTTCAAATTGAACACCG | 429 | Outer | 45 | 67 | 166 | 109 |
| MhL044A_00030 | AAAATGACGGAAGGTAAACTC/TTGTTGTTTTTGAATGCGTGA | 396 | Periplasmic | - | - | 4 | 3 |
| MhL044A_00033_342A | ATGAAAAATGCGTTACCTTATCT/TGTTAAGTAAAATCCGCCAAAG | 342 | Periplasmic | - | - | 135 | 169 |
| MhL044A_00033_343B | CGCACATTAGGCGAAAAATGGA/TTTATCTTCATTCAACACACGCTTA | 343 | Periplasmic | - | - | - | - |
| MhL044A_00041 | GATACCGATCAGCCGATTA/CTTGTTATCTTGAAGTTCATTTGGAA | 168 | Unknown | - | - | 173 | 202 |
| MhL044A_00066 | GGTGAACAAAGTGATGCAAGAG/TTTTTGAGGTGTGCAACC | 131 | Unknown | 95 | 119 | 178 | 165 |
| MhL044A_00067_359A | ATAAAGCAAACTGTATTAGCA/ATAGCCTAATTCCGTTGA | 359 | Outer | 64 | 74 | 86 | 91 |
| MhL044A_00067_360B | GATATGCAAACCTATACAGCAG/AAACTCCACTGATGCCATTAC | 360 | Outer | - | - | 122 | 148 |
| MhL044A_00068 | GCAGAAAAAATTGTTTCAACTGCG/TTTTAAACAAGGTTGAATAGTGGTG | 284 | Periplasmic | - | - | - | - |
| MhL044A_00074 | TGCTCGGTTTCAACGCAATC/TTGAGTGACCCAAAGATAATCTGCC | 278 | Unknown | 83 | 88 | 27 | 76 |
| MhL044A_00097 | TCAACAAAAATTGAACAAACC/ | 200 | Unknown | 111 | 110 | 128 | 142 |

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|---|---|---|---|---|---|---|---|
| | AGAATTTCTATTTTTTA GGATATCTA | | | | | | |
| MhL044A_00117 | TGCAGCTCGAACACCC CCGT/ ATTGGCGTGATGATGG GCAAAAT | 98 | Unknown | - | - | - | - |
| MhL044A_00156 | GCCGACCTACCTAATA TTACAA/ GAAATCTTCAAAATATT GTTGAATAG | 349 | Periplasmic | 60 | 69 | 83 | 86 |
| MhL044A_00167 | CTTTCTACTCCTGCCCC AGC/ GCCTCTCCAAGTGCGA ACTG | 184 | Unknown | - | 36 | - | 38 |
| MhL044A_00168 | GAAACAGCCACAGTTG CTCAG/ TTTTCCCCAATTCACAG AATGTT | 510 | Unknown | - | - | - | - |
| MhL044A_00169_335A | GACATTAAAGAAAGTG CGATTGTG/ GGTAATAATTTTAGTTT TTCCACCT | 335 | Cytoplasmic | - | - | 159 | 194 |
| MhL044A_00169_335B | GTGCGTGATAAAGTTC GTATTGAG/ TGACCAATCAAGCATC ATCTGT | 335 | Cytoplasmic | - | - | - | - |
| MhL044A_00180 | GTGACTTCTGCGGCGG TT/ CACTTTGGTTACTCCAC CTACATT | 196 | Periplasmic | 93 | 62 | 155 | 127 |
| MhL044A_00199 | GATGTTCAGCTTTCTCC ACT/ CTCGCCATTTTTTAATA ATAAATAGG | 386 | Unknown | - | - | 106 | 129 |
| MhL044A_00201_314A | ATGACTCAGCTTGATTC CG/ TTCACGGTCAAAGTTT ACATCT | 314 | Outer | - | - | 119 | 143 |
| MhL044A_00201_314B | GGAGAAGTGACACAA GCGGT/ TTTGTGCATTAATTGTA TCCCCTGCC | 314 | Outer | - | 122 | - | 149 |
| MhL044A_00214 | CAACCTAATATTTTTAC ACCAGA/ CTCTTTTTCTCCTTGTG TCAT | 293 | Periplasmic | 69 | 79 | 90 | 96 |
| MhL044A_00217 | GAAACAGTTACTCATC TTGATAC/ TTTACTTACTTTGAGAT CCATAC | 132 | Outer | 114 | 101 | 163 | 155 |
| MhL044A_00218_283A | GTGTTATACGACCGAG TAG/ GTATTTTTCAGACATAC TTCCC | 283 | Outer | 71 | 84 | 76 | 93 |
| MhL044A_00218_284B | TTAAAAGAGTTCGGGG TGTATG/ TGTATAGAATGGCTCA CCGTA | 284 | Outer | 50 | 45 | 101 | 74 |
| MhL044A_00220 | AATTGGACAAAAATTA ATGATGTG/ AACACAATATTTGGTG AGCATTA | 271 | Unknown | 72 | 80 | 94 | 100 |
| MhL044A_00233_406A | TCTATTGTACGTGGTG ATGTC/ TACATAATGTGGGTGG TGA | 406 | Extracellular | - | 24 | 70 | 52 |

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|---|---|---|---|---|---|---|---|
| MhL044A_00233_406B | TTAGAAAAAGAAAATAATGCAG/AAAAACCATTAAAATTAAGAAAG | 406 | Extracellular | 68 | 18 | 65 | 57 |
| MhL044A_00292 | TGCTCTAATGCAAACACACAAT/TGAGGTTAATGCTTTATCTACATC | 175 | Unknown | 43 | 81 | 59 | 70 |
| MhL044A_00297 | GAAACTAGCCCTTATGTA/TAAAGTGATAGGCTGTTTTG | 211 | Unknown | - | - | - | - |
| MhL044A_00301 | GAAGCAACATTACCCACAG/TTGCAAAATCACATAGAAATTTG | 464 | Periplasmic | 118 | 128 | 194 | 184 |
| MhL044A_00308 | ATCTCAAAAGCAGGACGA/TTCTGTGGCTTTCATTTCAGG | 218 | Periplasmic | 133 | - | 141 | 173 |
| MhL044A_00309 | GAAGAGGCGAAAACTGAATCAT/TTCTGCTTTTGGTGCTGCTAC | 500 | Periplasmic | 135 | - | 179 | 193 |
| MhL044A_00323 | TCTGAAGTCGAATTTAACCGC/AGGTTTATCCAATTTCTCAAG | 184 | Outer | - | - | 182 | 205 |
| MhL044A_00327 | GAGCCGTTTAAAGTAGTTACC/TTTGCTTCGTTCGAACCCTTC | 298 | Periplasmic | 12 | - | 32 | 26 |
| MhL044A_00334_401A | ATGAATAAGCATTGTTTTCGC/TTTCTGAATATTGCCACCC | 401 | Outer | 38 | 37 | 49 | 45 |
| MhL044A_00334_401E | CGGTTAGGCATTACCTTATC/TTCACTGCCCAATGTATTGC | 401 | Outer | - | - | 167 | 198 |
| MhL044A_00334_402B | ATCCAGCAAAGTGGCGAAACGG/TACAAACTGCGATGCAATAATCCCCT | 402 | Outer | - | - | - | - |
| MhL044A_00334_402C | AATGAAAAAGGCGGTATTTATATC/TGTTAAATTAAACCACGTCCATA | 402 | Outer | 25 | 21 | 26 | 29 |
| MhL044A_00334_402D | ATGACGCCTGAAATTATTCAAG/ATTGAATTTCTGCGCAAAGGTG | 402 | Outer | - | - | 148 | 185 |
| MhL044A_00334_402F | GGCAATAAAGCGAGCGTGA/CACCCCACTTGTCCCTCC | 402 | Outer | - | 68 | 33 | 58 |
| MhL044A_00334_402G | CTGGGCAATAAAAACGAAAGC/GGCATTAAAAGAACCTACAG | 402 | Outer | - | - | 137 | 172 |
| MhL044A_00334_402H | GATGTGGTGACAGCATTA/CATATCGGATTTGATTCGTTGA | 402 | Outer | 9 | 5 | 3 | 7 |
| MhL044A_00335_299A | CTTGTACAACAAGCACGCATTG/ | 299 | Outer | - | - | - | - |

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|---|---|---|---|---|---|---|---|
| | GGTAAAAGAGGTATA GAACAGGTC | | | | | | |
| MhL044A_00335_299B | CATTCGATGAAACAAA AAGGTGAT/ GGCAAACACGTCATAA CTAAA | 299 | Outer | - | 116 | 105 | 137 |
| MhL044A_00341 | GAGACAGGTAAAACAT ATCGTTT/ TTTGAAAATGATCTGG TTTTTTGG | 547 | Periplasmic | 30 | 138 | 100 | 107 |
| MhL044A_00349 | CAAACCATCACAGGAG CCGG/ GTAGAGCGAATTTCCT TGTCC | 346 | Periplasmic | - | - | - | - |
| MhL044A_00367_360A | GGGAAAGAACCATCAA AAAG/ GTTATCAAAAAATCGA GTGATAAA | 360 | Periplasmic | 51 | 71 | 66 | 73 |
| MhL044A_00367_360B | TTTGATAACCCAGAAC CAACAT/ TTTTCTTGTTAATTCCTC TTGATAG | 360 | Periplasmic | - | - | 56 | 66 |
| MhL044A_00375 | ATGCTGAAAAAAATCG GAC/ AGTAATAGTTACATCA AAGTTAAG | 347 | Periplasmic | 42 | 56 | 41 | 50 |
| MhL044A_00376 | AGTTCAGATACAACCT CTAAGTCTC/ TAAACGACGAGCCTGC CA | 173 | Unknown | - | - | - | - |
| MhL044A_00396 | GGAGAAACAACGGCC AGT/ TGGCGTTGTGGTCATG TTA | 103 | Unknown | - | 8 | 37 | 28 |
| MhL044A_00415 | GGCGGCAAAGTTACTG ATAATATT/ ATATACATTTGCAGTAC GAGGTG | 98 | Unknown | - | - | 183 | 206 |
| MhL044A_00453 | GCAGACACTTACATTA CTGAAAATC/ AGCCCAACCGCTGTTG TTACGTC | 203 | Unknown | 127 | 109 | 170 | 170 |
| MhL044A_00463 | AAAGATGTTACTATTCC AACTG/ TTTCTCAAATGCTGTTT TAATG | 299 | Unknown | - | - | - | - |
| MhL044A_00478 | AATGATGTTGTTAATAT CTCCAAAATT/ ATTTTCCTTGGTGACTT TCAGCA | 324 | Unknown | - | 16 | 17 | 20 |
| MhL044A_00482 | AATTTGCTCACTATTAG CGAG/ AGTGGTTTCTAGCTTG ATATTAC | 190 | Unknown | 41 | 22 | 43 | 37 |
| MhL044A_00490 | AATATAACCATTGAGC TTAAACAG/ AAAATAATTCGACCGC TTACAAA | 478 | Cytoplasmic | 54 | 42 | 68 | 64 |
| MhL044A_00491_345A | GAAACAACCTCTCAAG CAGAACTA/ TTTGACATCCCAATGG CGAGC | 345 | Outer | - | - | - | - |
| MhL044A_00491_346B | CAAGAAATCAACTTAG ATAACC/ ACTTAAATTGCGATCTT TTCC | 346 | Outer | 77 | 95 | 88 | 103 |

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|---|---|---|---|---|---|---|---|
| MhL044A_00500_395A | TCTGATTTTTGGAAAAGCGACCTTA/ATAGGCTAAAGAGGAATAACCAGC | 395 | Periplasmic | - | - | - | - |
| MhL044A_00500_396B | AAAGAAGGGAATTTATTTGTTG/ATTTAGACCGAAATTACTTTCA | 396 | Unknown | 17 | 20 | 20 | 24 |
| MhL044A_00536 | AAACGTGGCGGTGGGAG/TGGAATTAAGTTACATTGTAATGGCGTG | 189 | Unknown | 132 | 129 | 192 | 189 |
| MhL044A_00545 | GCTCCGGTAAATACGTTGATTA/CTCCGATAAACTCACCCCATA | 531 | Periplasmic | - | - | 120 | 145 |
| MhL044A_00566 | ACCTCACTGCGTTTTGGTTAT/TAACGCATCCACTTTTTTCAATAA | 328 | Periplasmic | - | - | 125 | 151 |
| MhL044A_00567 | GAAACCTCATTCCGCTTTG/CAATGCATCAATGGCTTTAACT | 327 | Periplasmic | - | - | - | - |
| MhL044A_00570 | CCGTTCGTACAAGAAGCAAA/CACGCCCCAAATCGCTTTGTA | 369 | Unknown | - | - | 81 | 98 |
| MhL044A_00578 | GATAAATTAGTGATTGCCCACCG/AAATTCCACGCCCGTATCAG | 357 | Unknown | - | - | - | - |
| MhL044A_00591 | ATGTCAAAGAAGAAAAAACA/ATGCTCTCTAATACTTGCTA | 367 | Outer | 67 | 60 | 78 | 81 |
| MhL044A_00592 | ATGAAACAAAAAAAATTAAATACTAC/AATTGTATTAGCTGTACCTG | 394 | Outer | 73 | 82 | 93 | 101 |
| MhL044A_00643_396A | GCACCTTTCGTAGTAAAAGA/ATCAACACTTTCATAGAAACC | 396 | Outer | 11 | 11 | 7 | 11 |
| MhL044A_00643_397B | ATGTCAATGCCAAATGTAGAA/GAATGAACTACCAATGTTGAA | 397 | Outer | 59 | 75 | 72 | 83 |
| MhL044A_00644 | GATACTATTGGTTTCGTAGATC/TTTTTTCGCTTCTTCTGGTTTAG | 263 | Outer | 65 | 44 | 67 | 68 |
| MhL044A_00653 | GCCGCTGAAAAAATAGTC/TTTTACAATTTCAAATTCTAAACCT | 158 | Unknown | 57 | 83 | 92 | 94 |
| MhL044A_00663 | GAATCGTTCAGAACATTAACC/TAAGCCCGCCATTTTGATAAT | 162 | Outer | - | - | - | - |
| MhL044A_00670 | GTACATTTTAGACCGCTTGGCA/GCCGTTTGGTGTAACAGTTGTG | 159 | Extracellular | - | - | 185 | 207 |
| MhL044A_00720 | AAAGATGAGCTTGTGGTATTTAG/ | 312 | Periplasmic | - | - | 54 | 62 |

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|---|---|---|---|---|---|---|---|
| | TTCGCTGATTTCTGCAAAAC | | | | | | |
| MhL044A_00752 | CGTTTAACCGTTTATTGTAGTG/TTTCGCTAATTTTACTTCATTTACCC | 344 | Periplasmic | - | 134 | 52 | 110 |
| MhL044A_00780_305A | CAAAGGCTGTCTGACGTAGAT/GCCTATCCCAATTTCCACTTCAT | 305 | Outer | - | - | - | - |
| MhL044A_00780_306B | CTTAACTGGAAGAAACCTTGG/ATCGGAACCTAAACCAATATAAA | 306 | Outer | 62 | 25 | 28 | 40 |
| MhL044A_00782 | CAGATTCAGCAAAAAATCCAAC/AATCGGCGTGCCTTTA | 387 | Outer | 63 | - | 85 | 89 |
| MhL044A_00790 | GCAAATGAAGTGAATGTTTATTCTTA/TTTTTTCTCAGAGAAATCGTCAAA | 342 | Periplasmic | 130 | - | 144 | 174 |
| MhL044A_00809 | GCGTGTAATGAAGAAAAAGCG/TTTTGCTGCTTTTAACTCTTGGT | 364 | Periplasmic | - | - | - | - |
| MhL044A_00814 | ACGCATAAAAACAGCGATGAGC/GCCGTCCCAAGCTTTAATTGC | 273 | Outer | 101 | 29 | 16 | 54 |
| MhL044A_00822 | TACTTATTGTTGTTTTGGGTTA/ATTGTTCATAATTTGCTTAGCCG | 327 | Periplasmic | 49 | 57 | 61 | 65 |
| MhL044A_00827 | GAAGAAAGAGTGGTGGCGTTAG/TTGACCGACATATTGAATATTGGC | 314 | Periplasmic | - | 10 | 5 | 9 |
| MhL044A_00839 | CAGTTTGAACACAACGAATCC/AACACCGATAAATGACATACCTG | 527 | Periplasmic | 1 | 3 | 10 | 6 |
| MhL044A_00843 | ATGAATATCTTACAAGCAGGCG/GCGACCCCGTAGATAATCC | 155 | Periplasmic | - | - | - | - |
| MhL044A_00855 | GTACCGCCGGAAAAAACA/AATTTTACATAGTGAGTGGTG | 197 | Unknown | 128 | 90 | 189 | 171 |
| MhL044A_00867 | GGTGAATCCACTTCTGCGGT/ACGAACACGTAAGACTTGCC | 166 | Outer | 119 | 115 | 150 | 160 |
| MhL044A_00869 | GTGAATGCTTCCGGTAATAAC/TTTTCTTGGTAAATAACGAGCC | 426 | Outer | - | - | - | - |
| MhL044A_00870 | ACACCAACTAGCCCAAGC/GTAAATTTTCCAACCTTCAGCTT | 122 | Unknown | - | - | - | - |
| MhL044A_00876 | CTACTTGAGCGTATAAACAACAAA/TTGTACACTAATATCTTTACCGAA | 257 | Periplasmic | 84 | 31 | 40 | 60 |

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| MhL044A_00931 | ATGTCTAAATGTCCTGTTACAC/GAGTAAACCCGGTTGACCTA | 502 | Periplasmic | - | - | 127 | 158 |
| MhL044A_00964 | ACAACAGAAAGCATTAACCAA/TTTACGGGCGTTATAAATCTT | 251 | Unknown | 32 | 49 | 42 | 44 |
| MhL044A_00981 | AGCAATAAATCGACTAAGAAGCC/ACGAATATTGCGATATTTCTCTC | 363 | Unknown | - | - | 126 | 153 |
| MhL044A_00991 | GATATCAAATTTGTGATGGAGC/CTTGCCGCCCATCCATTTAT | 238 | Periplasmic | 16 | - | 18 | 21 |
| MhL044A_00993 | ATGGGAACTAGACTTACAACCCT/AGCTGCTCTAGCAAATTGAAGAG | 953 | Cytoplasmic | - | - | - | - |
| MhL044A_00993_476A | ATGGGAACTAGACTTACAACC/AATGTTGTTATCCCATTGCTGC | 476 | Extracellular | - | - | - | - |
| MhL044A_00993_477B | GGTGATTTAGCTGGTATTA /TCTAGCAAATTGAAGAGAAGAT | 477 | Extracellular | 31 | 32 | 57 | 43 |
| MhL044A_01009 | GATCTAAAAATCGGTATGTCAATT/TACTGCATCTTTGGTATGGAA | 333 | Periplasmic | - | - | - | - |
| MhL044A_01030 | ATTGAAACGAAACAAGCAGAG/AATGACTTTTGCAGTATATTTGAG | 102 | Unknown | 106 | 94 | 157 | 144 |
| MhL044A_01032 | CAACAGAACAATTTCCACACCA/TTCATAGCCAAATTCTTTCGCCTC | 368 | Unknown | - | - | - | - |
| MhL044A_01039 | ACTTCCGTTTACCAAGGC/TTCCAGCTCAACACCATA | 219 | Periplasmic | - | - | 80 | 97 |
| MhL044A_01040 | CATCACGAAATCAAAATGTTAGAT/AACTTGCTCTAAATATTCAAATAAAC | 149 | Periplasmic | - | - | - | - |
| MhL044A_01046_490A | GCAAAAAATTCTGAAAAAACGACC/TTTCTTGTCGGTTTTGGCAGTAT | 490 | Periplasmic | 44 | - | - | 46 |
| MhL044A_01046_491B | TTTGAAGCAGGTTATTCGGTG/CTTAATCTCAAACTCTCGTTGTAG | 491 | Periplasmic | - | - | - | - |
| MhL044A_01047 | GATGATAAAAGCAACGAAAATAAA/GTAAGTATATCCGAACGGACTT | 548 | Periplasmic | 74 | 39 | 71 | 71 |
| MhL044A_01048_330A | TTGGTAAAACAAGCGACGAG/TACTTTATCCAACATATAATCCAC | 330 | Outer | - | - | - | - |
| MhL044A_01048_330B | CGTGGGAGAAATAATACGGACG/ | 330 | Outer | 22 | 65 | 200 | 113 |

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|---|---|---|---|---|---|---|---|
| | GTAAGCCGCACTGAGTTTCACAT | | | | | | |
| MhL044A_01049_281A | GCAGTGAATGTGCCGGAT/AATCAAGCTCGAATAATCCAAA | 281 | Outer | - | - | 145 | 182 |
| MhL044A_01049_281B | TTGGATTATTCGAGCTTGATTG/GAAGGTTTTGATGGCGGAG | 281 | Outer | 39 | - | 60 | 55 |
| MhL044A_01050_469A | CAAGGTGAAAGGGTGCGT/AAAGAATAAACGCCCGTTTGCG | 469 | Extracellular | 80 | 126 | - | 126 |
| MhL044A_01050_469B | AACTTGCCGAATGACACCGA/TCCGACAATAACTTTAGCATTGGTA | 469 | Extracellular | - | - | - | - |
| MhL044A_01070 | AACGGTTCTTCACAAGCAAAT/AACATCTAACTCGGTTTTATTGAAAC | 120 | Unknown | 131 | 133 | 165 | 179 |
| MhL044A_01074_416A | ATGGAACTCAATCGTAGAGA/AATAAATGTACCCACTTCACGG | 416 | Periplasmic | - | - | 75 | 92 |
| MhL044A_01074_416B | GAAGTGGGTACATTTATTCACC/TTTAACCGCACATTTTTTGAAG | 416 | Periplasmic | - | - | 102 | 122 |
| MhL044A_01077 | GAAGCCCCTAAGTTATCC/TTTAGCTTGAGATTCACGTACAG | 142 | Periplasmic | - | - | 121 | 147 |
| MhL044A_01082_405A | ATGTCTGTGATGGCTGCCG/ACCGATAGAGCTGTAATGGTGAG | 405 | Periplasmic | 14 | 12 | 15 | 14 |
| MhL044A_01082_405B | TACGACAACAAAGATTACAAAGGCTA/TACCTCAATCGCACCGTTAAA | 405 | Periplasmic | - | - | - | - |
| MhL044A_01086 | GAGTTTCAGCAAGATCCCAAAG/AAATAGTTTCTCTAAGGTCATAAAG | 464 | Outer | - | - | 130 | 163 |
| MhL044A_01093 | ACGCAATGGGAGAAAATC/GCCTTGTTCAGATAAAATCAT | 275 | Periplasmic | 70 | 51 | 89 | 85 |
| MhL044A_01129 | AATATAACCGCTTCAACTGAG/TTGATTTTTCTTAGTGAGTGCATTT | 149 | Unknown | 126 | 113 | 152 | 164 |
| MhL044A_01130 | GGCCAAAAAGGCAAAACAACC/TAATACCCAAACACGGCCGTAAT | 368 | Outer | - | 100 | - | 121 |
| MhL044A_01152_388A | AAGCAACAATGTTTACTTGG/AAAATCAATTTGTGGCAGAACG | 388 | Outer | - | 106 | 69 | 104 |
| MhL044A_01152_389B | GTTCTGCCACAAATTGATTTTAACT/GCTATACGGCTCGGTGTA | 389 | Outer | - | 125 | 184 | 191 |

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|---|---|---|---|---|---|---|---|
| MhL044A_01182 | GCATGTAGCAGCTCATCAGAT/GTATTCTAATACTGCACGACGG | 161 | Outer | - | - | - | - |
| MhL044A_01183 | TCTGATGTGCGAATTTCTATTGA/TTTAGTTAAGTATGGTGACCATG | 428 | Periplasmic | - | - | - | - |
| MhL044A_01192 | AGTGTTAAAACCTCATCACTTACT/TTGATGTTTTTGTTTAAGGCTG | 108 | Unknown | - | 117 | 187 | 190 |
| MhL044A_01223_751A | CTTCAATACTACCGTGATTT/AAGTAGCACTTTACCTCCT | 751 | Outer | 20 | 23 | 47 | 33 |
| MhL044A_01223_752B | AGACCGACCCCTCACGCTTA/GCTATATCCCAGTTTGAAGCCGG | 752 | Outer | - | 14 | - | 15 |
| MhL044A_01226 | AAAGAGTATATTGAGGTGCGTA/TTTCTTACTCAAGAAGACCAC | 212 | Periplasmic | 29 | 7 | 12 | 19 |
| MhL044A_01255_350A | GTGGCTCAAACTGCTTCAA/GTTTACGTTTGCACCGTGG | 350 | Outer | - | - | 202 | 215 |
| MhL044A_01255_350B | GATTCTGCCCTTCACGAAAATGT/TCCACGAGCTGATAATTGACCAC | 350 | Outer | - | - | - | - |
| MhL044A_01263 | AAATCTGATGAAATCGCTGTGA/GTAAAGAACACCTTTCGCTT | 315 | Unknown | - | - | - | - |
| MhL044A_01272 | GCTGAAACATTTGTGACTATC/CTTAATCCAACCTTTCTCTTTAT | 320 | Unknown | 13 | 19 | 14 | 17 |
| MhL044A_01284 | GATGAAGGTAATAACACACAGG/TAATATCATTGAAGCCGTTGGTC | 212 | Unknown | - | - | 171 | 201 |
| MhL044A_01319 | GCGGAAGGTTTGCTACTTACCC/ATCAATTTCTAACGCCCCCAAAC | 342 | Periplasmic | - | - | - | - |
| MhL044A_01388_291A | CCAAGTATTCCTCAAGAGC/AACCACATCACATTCATTATTTA | 291 | Periplasmic | 100 | 98 | 139 | 138 |
| MhL044A_01388_292B | CCTAGCCAATTATCAGTGTTA/GTTTCCTTTTTTATGCCCC | 292 | Periplasmic | 94 | 86 | 118 | 120 |
| MhL044A_01397 | GGTAACTTAAGTAAAGTGAGTG/GCCATCATATGCTGGGGTTTG | 284 | Outer | - | - | 48 | 53 |
| MhL044A_01399 | TCAGATGCAAAACTAAAAAGCCAG/CTCACTTAACATTTTCGCTAATTC | 227 | Periplasmic | - | - | 131 | 166 |
| MhL044A_01408 | TTTCTCACCCACATTCATTTGTT/ | 212 | Extracellular | - | - | - | - |

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|---|---|---|---|---|---|---|---|
| | ATCGCATATAATCTCCACTTTTT | | | | | | |
| MhL044A_01413 | GGTATTGAAACCTCTTGTGATGAA/AATCGGTGGTAATTCGGTC | 343 | Extracellular | 28 | 43 | 134 | 82 |
| MhL044A_01414 | AAAGAATTAGAACAATCCAACG/GAATTGTTCGCCATACTTCAG | 259 | Outer | 109 | 89 | 97 | 118 |
| MhL044A_01424 | TTAAATACCTTGCCTGC/AAACAACGCCATATAAAACT | 481 | Periplasmic | 33 | 35 | 38 | 36 |
| MhL044A_01458 | CAAATTAAGCAATTACCGCC/TTCCACTTTCCATTTACTG | 139 | Cytoplasmic | 92 | 102 | 132 | 133 |
| MhL044A_01473 | GCTCCACAAGCTAACACTT/CATAGTTACTTCTTTTGAACCTTG | 378 | Outer | 113 | 73 | 84 | 108 |
| MhL044A_01477 | ACTTTAGCAGGAAGCCCGAT/TAATACCGCTAACGCCGCCT | 165 | Periplasmic | 6 | 63 | 50 | 42 |
| MhL044A_01479 | CGACCGCAACTGAACATTCC/GCTAAATTGACCCATCGTACCT | 474 | Periplasmic | - | - | - | - |
| MhL044A_01483 | GTGATTTTAGGTGCGGCACT/AATTCTAATCCCAGCAGTCACAAC | 238 | Outer | 123 | 124 | 186 | 181 |
| MhL044A_01484_408A | AGATATTATCCTTCCGCCAC/AACAACAGAAATCCAACCTTTTT | 408 | Outer | - | - | - | - |
| MhL044A_01484_408B | GTAGCAACCAGAACTAATCAGGGA/AGCAGTCTCACCTACATTATCCA | 408 | Outer | - | - | 197 | 212 |
| MhL044A_01491 | GAGGTAAACATTAAACTACTTGGT/ATGAGGACATTCCGGTGT | 353 | Periplasmic | - | - | 169 | 200 |
| MhL044A_01498 | GATATTGCCGATGTCAAACCG/TTTAATTAATTTTTCCGCTTTGGCAA | 150 | Unknown | - | - | - | - |
| MhL044A_01531 | AGTGCCGAGCTGATTGTC/AAGCAGATCATCATCGTCCTCC | 143 | Unknown | - | - | - | - |
| MhL044A_01536 | AATATACCAACTTCCCACTAC/ATCCTTTTTAATCCTTAATTTACC | 225 | Periplasmic | 112 | 97 | 153 | 146 |
| MhL044A_01541 | GCTGATTATTTGAGCCAAGAC/CTTAAAATCCCCCAACTCA | 372 | Unknown | 40 | 64 | 36 | 51 |
| MhL044A_01548_276A | GAAACCCACCAAATCGGCA/GAGACGAACCCAATCCAGCAC | 276 | Cytoplasmic | 58 | 17 | 24 | 34 |

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|---|---|---|---|---|---|---|---|
| MhL044A_01548_276B | CAACAAAAAAGCCTGTGGGCAA/GAGTAAAAGCACTACCTTCGCC | 276 | Cytoplasmic | - | 59 | 103 | 99 |
| MhL044A_01561 | GATGAAAAAGCACAAGCACCT/GTTAAATAATTTTAACGCTTCTTGG | 276 | Unknown | - | - | - | - |
| MhL044A_01562 | TGTAATGATAAAGCCGAAAAGTT/TTTTATCATCGCCCCTTTAAAC | 263 | Cytoplasmic | - | - | - | - |
| MhL044A_01564 | ATGAAAGTTGGATTAGTGCTG/TTTGGCTAAATATGCCCTTAAA | 280 | Extracellular | 47 | 28 | 112 | 72 |
| MhL044A_01566 | AAATTAGTTTCTTTGATTGTCGT/GCAATCCATTGGTTGA | 237 | Periplasmic | 37 | 55 | 44 | 48 |
| MhL044A_01570 | GAAGGTCGTTTAACTGTTTATTG/GTTTGCTAATTTAATTTCCGCC | 345 | Periplasmic | 78 | 58 | 114 | 102 |
| MhL044A_01577 | GTGCAATTAAATCAAGCATTACCT/TTGCGTTAATGTTTTCACCAA | 185 | Unknown | 2 | - | 11 | 8 |
| MhL044A_01631 | ATGTTTTCTTTTTATGGTAAGA/CCACTCATATCCAACTTTG | 184 | Extracellular | 48 | 47 | 63 | 61 |
| MhL044A_01638 | AAATCGCCCGAGTTAATGC / ATCATTTCTTATTCGCCAAAACGT | 274 | Unknown | - | - | - | - |
| MhL044A_01650 | CAAGAAATTAAACTTTTGAACAGA/TTCTTCATTTTTAGCTGGAGGT | 272 | Periplasmic | 103 | 66 | 162 | 136 |
| MhL044A_01656 | GAGACTATCGCTCTTGCAGTA/AATAACCTTTAAATCAACCGGAA | 292 | Periplasmic | - | - | - | - |
| MhL044A_01672 | ATGTGGCGAATTTTTATATATTTCT/TTTTGATTTTTTGCGGTTGCT | 437 | Outer | 90 | 120 | 77 | 112 |
| MhL044A_01681 | CAAGAAACTATCACTTTTGCG/TTTCCCAGCCATCCAAGTATTA | 241 | Periplasmic | - | - | 45 | 47 |
| MhL044A_01683 | CTTTTCTCTCAACCCAAATTC/AGATTGGCTCATTTCATTG | 587 | Cytoplasmic | 107 | 91 | 123 | 130 |
| MhL044A_01687 | AATCAACTGATTGGGCAATGG/CTGTTTTATACATTGCCCTTTAT | 166 | Unknown | - | - | 19 | 23 |
| MhL044A_01700_465A | GCGACTGAAAATAAAAAAATAGAAGA/ACAATGCAAGCGATGTA | 465 | Outer | 61 | 72 | 74 | 84 |
| MhL044A_01700_465B | GATTATCCTGTGGTAGATAAAAAT/AAACTTCATTTCAAATG | 465 | Outer | 85 | 87 | 91 | 105 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | CTAAAC | | | | | | |
| MhL044A_01701_292A | GATGTTCAATCTGCCA AAGTTC/ ATAAGGATCATCGGTA TTTTTTGCGT | 292 | Outer | 140 | - | 156 | 186 |
| MhL044A_01701_293B | TTTGGTAAAAGCTCTA CGACAC/ TTCTATTTGACGTTTTC CGC | 293 | Outer | 21 | 33 | 9 | 25 |
| MhL044A_01714 | AACTCGCAAGGCGATG ATCC/ TCCATCATAAATCGGTT TAAGCAC | 179 | Cytoplasmic | - | - | - | - |
| MhL044A_01740 | TCAAACAGTTACACCA CGGAAAC/ TTGAGAACTGGATGCC G | 204 | Unknown | - | - | 198 | 213 |
| MhL044A_01744 | GAGGATATGATTCAGG AAAATAAA/ GTGGCGTTTTTGCTGA AGTAA | 446 | Unknown | - | - | - | - |
| MhL044A_01777 | GATGTATTAACCAGTA TTAAGCC/ TAAACATTGGCTAAAT TCGTCAG | 324 | Periplasmic | 116 | 107 | 160 | 159 |
| MhL044A_01802_405A | CACTGGGTTGATATTA AAAACTCC/ CAAGCGGAACACTTCG GTTT | 405 | Periplasmic | - | - | - | - |
| MhL044A_01802_406B | TTTGCGGAAGAAGATG GTTCA/ AGCCCCCACAATTTTCT CAATG | 406 | Periplasmic | - | - | - | - |
| MhL044A_01804 | GCACCGCGTGAGTATA AGC/ GATACTCGCTTGGTTG TCGGT | 195 | Periplasmic | 110 | - | 191 | 188 |
| MhL044A_01831 | CAAGTGCAGGCTGAAA AAGCAG/ TTTCAACCCGGCTTTAA TCAGATC | 117 | Unknown | - | - | - | - |
| MhL044A_01858_294A | ATGAAAAGTGCAGGA GGTTATAC/ TGCATTTGGAATACGG AAG | 294 | Outer | 139 | - | 154 | 183 |
| MhL044A_01858_294B | TTTGATTACCGTGATAA TGCTGC/ GTTACGTCCAGGTTCA TAATAGC | 294 | Outer | - | - | 174 | 203 |
| MhL044A_01859 | GAAGAAACAACAGCG GAATTA/ CTTCTGAATCAGGAAA AGCGAG | 127 | Outer | - | - | 190 | 208 |
| MhL044A_01863 | GAAGGAACAACGCTTG CAGAAAA/ TTTCACGATGTAGAGG TTACGC | 544 | Periplasmic | - | - | - | - |
| MhL044A_01901 | AAAGCAGATCCTAAAT TTGTGG/ TTTCTTAGCATCTGCTG CTTTA | 241 | Periplasmic | - | - | - | - |
| MhL044A_01906 | GAAAATAACACTTGGG TAGACA/ GAAGATAGCCTCTAAA CGTAC | 314 | Unknown | 75 | 76 | 53 | 80 |

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|---|---|---|---|---|---|---|---|
| MhL044A_01917 | CAAGCAGGTGATGTGATATTCC/GAACTTCACCCCGAAACCGGC | 223 | Outer | - | - | - | - |
| MhL044A_01943 | ACCACCGTTTACGATGCAGAA/GTATACACGCATACCAACAC | 372 | Outer | - | - | 161 | 195 |
| MhL044A_01953 | TCAACAGAGGTGCAACAAC/TTTTTCAATGCGGATCTTCAAC | 210 | Unknown | - | - | - | - |
| MhL044A_01967 | ATGCAGAAAAAATCACTCATATC/ATACAACACGTCTGCCAGC | 171 | Periplasmic | 117 | - | 136 | 156 |
| MhL044A_01970 | GCTTGTAGCAGCAATGAACCG/GAGAAGTAACATACTGCCCCATT | 187 | Unknown | - | 132 | - | 167 |
| MhL044A_01972 | GAAACCACACCGGCTCAACTTC/GAACGTATGTGAAAACTCAATATAG | 484 | Unknown | 97 | - | 113 | 128 |
| MhL044A_01978 | ACTATCATTAAGATTGGTCACT/TTTTGAAATAACATCTTTAGCC | 327 | Periplasmic | 18 | 27 | 22 | 27 |
| MhL044A_01987 | GCGACAAAGAATACAAAAG/ATTCATTACATAAAACTGATTAGTC | 219 | Unknown | 115 | 114 | 151 | 157 |
| MhL044A_01988 | AACACTAACTTCAATTTATCGG/ATCGAACAAGCTCTTCACA | 392 | Cytoplasmic | 19 | 85 | 95 | 78 |
| MhL044A_01992 | ATCACTGATGTATTAGGGCGT/TTGCATAAAGAATGTGCCTTTTG | 367 | Unknown | 36 | 46 | 133 | 87 |
| MhL044A_02017 | ACAGAACAAGAGCAATTAGCC/TTTGCTATAATCATCACTTAATGTAC | 108 | Unknown | 138 | - | 199 | 199 |
| MhL044A_02039_635A | GGTGATAATGGTCAAACAATC/TTTATTACCTGCATTGATACCATCT | 635 | Extracellular | - | - | 13 | 13 |
| MhL044A_02039_636B | AATGTTGCGTCTAATCTGATACC/CTGGTATCCAACGCCTACAC | 636 | Extracellular | - | - | 201 | 214 |
| MhL044A_02040 | GTGGATACCGGTTCTATCACGGT/GGTGCACCAACAGTTGCTAAAC | 161 | Extracellular | - | - | 129 | 161 |
| MhL044A_02044 | ATGAACCAATTCTTCCCTCTAATC/TTTTCTATACCGCTCATCCGTC | 384 | Periplasmic | - | - | 164 | 197 |
| MhL044A_02047 | TGTTCATCAAGCATTAACCCAG/ATCAATATTCTTCAACTCTTCTTCCG | 251 | Outer | - | 137 | 188 | 196 |
| MhL044A_02068 | ATGATGGCAGAAACCACTTCTCG/ | 398 | Periplasmic | 35 | 41 | 35 | 39 |

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|---|---|---|---|---|---|---|---|
| | ATCAAACAACGCTTTG CCAAGG | | | | | | |
| MhL044A_02078 | CAAGATAAAATTCAAA ATGTAGCA/ AGTTTGTTTAGCTTCTT TGAT | 319 | Unknown | - | - | - | - |
| MhL044A_02092 | GCTCCTGTCGGAAATA CATTTAC/ GAAACGATAAGTTGCA TTCGCG | 172 | Unknown | - | - | 180 | 204 |
| MhL044A_02104 | TGGTTGATTCCATTAAC TGTCG/ GAAGCGTCTTTCAAAT GGAT | 524 | Periplasmic | - | - | - | - |
| MhL044A_02106 | AAAAGGAAAAGCACAC CACGG/ ATCATATTGTCTATACG GATTTG | 291 | Periplasmic | 108 | 112 | 158 | 154 |
| MhL044A_02132 | CAAGAGATTACCGTGT TTGCTG/ ATTTACAGGATAAAAC CCAGCTGC | 251 | Periplasmic | 3 | - | 6 | 5 |
| MhL044A_02137_414A | ATGAAAAAACAAGACA ATATTGAC/ GTTTGAGTAATGGTAG CTTAAA | 414 | Periplasmic | 56 | 50 | 62 | 67 |
| MhL044A_02137_414B | TTAGGCTCTATCACAG CAAATC/ TTGTGTGTATTTAGGCT CTTTA | 414 | Periplasmic | - | 136 | 149 | 176 |
| MhL044A_02139 | GCCACCAGCTCAACAG AA/ ATGGGAACTGCCTGCT ACATC | 371 | Cytoplasmic | 53 | 131 | 110 | 116 |
| MhL044A_02192_691A | GCAACCTATTCACCAA CCTAT/ ATAGTTAGCACGTTGA ATGCC | 691 | Unknown | - | - | 46 | 49 |
| MhL044A_02192_692B | GATAATTCAGGCTCTA GCGGTGT/ TTGATAACCCATGCCA ATACTACC | 692 | Unknown | - | - | - | - |
| MhL044A_02221 | AAAACCATTGTGGCGA TTGATG/ ACCTACCGTGATCTGA TAAT | 398 | Outer | 99 | - | 87 | 111 |
| MhL044A_02240 | TATCCCCTTTTATTTTC GCTG/ AAATAGCTTTTCTAAA GTGAAAAAGA | 469 | Outer | 7 | 4 | 2 | 4 |
| MhL044A_02243 | ACAGGGAATATGAGCA ATGA/ TCTTCTGTATTTACCTG CC | 465 | Outer | 27 | 38 | 25 | 32 |
| MhL044A_02244 | GAGCAGAGCCAATACC AAC/ TTTCGGCACAGTTACA CGATTTA | 281 | Unknown | - | - | - | - |
| MhL044A_02245_515A | GTGAACAATAAACGCA GAAATC/ GATGGCATTGGCTTTA TGA | 515 | Periplasmic | 76 | 26 | 99 | 79 |
| MhL044A_02245_516B | TTTTATACCAGTTGGGC GATTT/ GGCTAATTTCTCTACCT TCGC | 516 | Periplasmic | - | - | 51 | 59 |

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|---|---|---|---|---|---|---|---|
| MhL044A_02275 | TCGGATTGGGAAATAATAGAAG/CTTACAATATCTATTTTGCATAGC | 152 | Unknown | 89 | 92 | 116 | 119 |
| MhL044A_02282 | GAATCAATAGAGAATCCACAAC/GAAACTAAAGCCAACATTTACT | 954 | Cytoplasmic | 4 | 1 | 1 | 1 |
| MhL044A_02282_467A | ACAGAATCAATAGAGAATCCACA/TTTACCGTTTAAGGCCAAT | 467 | Outer | - | - | 98 | 115 |
| MhL044A_02282_467B | AAAACAATCAGTAACCATGCAA/GAAACTAAAGCCAACATTTACT | 467 | Outer | 88 | 53 | 58 | 77 |
| MhL044A_02291 | CAACAAGCTGAACGTAAA/GCAATAATAGCAGTAACGCCAG | 127 | Unknown | 87 | 105 | 115 | 124 |
| MhL044A_02292 | GGGACGTTAAGTACATCATC/TTTTATATTTTTCTTCGCCCATTC | 202 | Unknown | 105 | 61 | 31 | 75 |
| MhL044A_02301 | AACACTAACTTCAATTTATCGG/CACACTTCCTGAAGCG | 239 | Extracellular | - | - | - | - |
| MhL044A_02304 | GGCAAAATGACCCAAACGGT/ATCTAGTTCTTCAAAGAATCCATCTT | 328 | Unknown | - | - | - | - |
| MhL044A_02332 | AACGAAACTGGAGAAAGTTG/AAAATGTTTCCACTCTAACCC | 198 | Unknown | 104 | 96 | 147 | 140 |
| MhL044A_02351 | ACCTTTGTTTACTGCTTAGAGGC/GTAGAAGTTATGTAAGCTGAACGG | 533 | Periplasmic | 10 | - | - | 12 |
| MhL044A_02362 | GCCGAACGCCAACTACAATG/ATTCCTATTTTTCTGTTCTTGCCG | 222 | Unknown | - | - | 196 | 211 |
| MhL044A_02372 | AACACATTAACATTCTCTACC/TTTGAACTGGTGAATTGCAT | 195 | Unknown | 55 | 52 | 55 | 63 |
| MhL044A_02386_395A | GAAAATAACGATGTAGCGGTG/GCCGTAATCCTGATCTTTTTC | 395 | Outer | 134 | - | 176 | 192 |
| MhL044A_02386_395B | TTCAAAAATCGGGGCAAAAATAC/TTTCATCTCCATTCCCAACATA | 395 | Outer | 125 | - | 140 | 168 |
| MhL044A_02387 | CAAGGTCCATCTATTGTTGCAGAC/TTTCGCCGCTTTGCTTAC | 485 | Outer | - | - | - | - |
| MhL044A_02388 | ATGAAAAAAATTGCCTTAC/TTTATCGGTGTAGGCATAG | 470 | Outer | - | - | - | - |
| MhL044A_02398 | CAAACACCGCCAACTACAATGCC/TTCTACCGTTGCTTGCC | 112 | Cytoplasmic | - | 2 | - | 2 |

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|---|---|---|---|---|---|---|---|
| | CAATTCC | | | | | | |
| MhL044A_02399 | ATGACACAAAATAATTTTCGCAAAG/TAACTTATTCAGCCAAGCTAAAAA | 439 | Extracellular | - | - | - | - |
| MhL044A_02400 | GCTTGTAGTACAGTGGTTACCC/TATGCAATTTGCCGCTAACGTTTTT | 138 | Unknown | - | - | - | - |
| MhL044A_02410_392A | ATGAACACAGCATTAGAAAAAG/ATTGATTGCCCCTAAACGAA | 392 | Periplasmic | - | - | 39 | 41 |
| MhL044A_02410_393B | CCCGATATGCCGTTTTTCCC/TTCAGGTGCATCTCCCACATATT | 393 | Periplasmic | 5 | 13 | 29 | 18 |
| MhL044A_02413 | CACGCATTATCGGCTCAATTA/TCTCACACTGATATAAATTAACGC | 130 | Cytoplasmic | - | - | 96 | 114 |
| MhL044A_02444 | GATGCTCAATCTGTTGCAGAAT/TTTTTTCGATTTTTTACCGCCAC | 213 | Periplasmic | - | - | - | - |
| MhL044A_02471 | CAATCTGAAAGCAGAGTGGG/AGGTAAAAATTGCCCTAAATTTTCC | 330 | Periplasmic | 26 | 48 | 146 | 88 |
| MhL044A_02476 | GCACCTAAATCTTTTACTTACTGTT/TCTAGGACAGCCCCTAAAAGT | 295 | Periplasmic | - | - | - | - |
| MhL044A_02480 | ATGAAGAAAGCAGTATTAAGTGC/TTTTAAAAATTCAGATAAGTTGTC | 329 | Periplasmic | 34 | 30 | 23 | 30 |
| MhL044A_02503_329A | AAAAATAAAGCTGGCTTGCGTG/TGCTCTACCGTCCACAACG | 329 | Periplasmic | - | - | - | - |
| MhL044A_02503_329B | AATACGGAAGAGAAAAAAGCAAC/TAAATCAATACGGTAAACTGCGA | 329 | Periplasmic | - | - | 34 | 35 |
| MhL044A_02512 | GCCGCATTCCAATTAGCTGAAA/GAATTTGTAGTTTACGTTTAAACCG | 493 | Outer | - | 111 | 107 | 134 |
| MhL044A_02530 | TCGAATAAAAGCGGTTGGACA/TTTGGTTTTACCTTTAGATTGCG | 183 | Unknown | - | - | - | - |
| MhL044A_02542 | AGCCCCCTTAAGAAAGAAGA/AATTAAGTTTTCACTCGGCAC | 126 | Unknown | 137 | 139 | 175 | 187 |
| MhL044A_02553 | GGCAAAGAATATATCGAAGTGC/TAAGAACACAACCGCATCAAC | 212 | Periplasmic | 15 | 15 | 21 | 22 |
| MhL044A_02556_699A | TCTGTTGTACGTAATGATGTGG/AGCAGTAAAATGGCTGTCTT | 699 | Outer | - | - | - | - |

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|---|---|---|---|---|---|---|---|
| MhL044A_02556_699B | AATATCACGGTAAATAAAATGGC/ ATATCCTAGCTTAACACCAGC | 699 | Outer | 46 | 40 | 64 | 56 |
| MhL044A_02584 | GACGTGTTTAGCGGAAGCG/ AAGCAGAGAAACATTCAGGTC | 154 | Outer | - | - | 193 | 209 |
| MhL044A_02624 | AATGTAGTGTATTCTTGCACAAC/ GGCTGCAAATCCAGATTTTCTC | 143 | Unknown | - | 118 | 168 | 180 |
| MhL044A_02625_350A | AATACCGTTAATGTTGGCAATAGC/ ATTGAGTTGTTCCGGCGTTAAA | 350 | Unknown | - | - | 195 | 210 |
| MhL044A_02625_350B | AGTACATCAACGGTCAGTGTAACAA/ GATAATGTAATCGGCTTCCTGGT | 350 | Unknown | - | - | - | - |
| MhL044A_02626 | AGTCCTGCTAATAAACCCTC/ ATGAGTATGTGTACTTTTCCAG | 92 | Unknown | 86 | 104 | 177 | 150 |
| MhL044A_02654 | GGACCAAAAGTAGCTTATACCCA/ ATAACTTGAATTGACAACAACTTC | 232 | Unknown | - | - | 109 | 135 |
| MhL044A_02682 | TCATACTCTGAACATGCGG/ TCGTTTCAAGCCTTGCTGATA | 107 | Unknown | - | - | - | - |
| MhL044A_02713 | GATCATAAAGAAACCGGTACAC/ CCAATATGCTCGTAAGCCTAC | 364 | Outer | 136 | - | 143 | 175 |
| MhL044A_02723 | AAGAATGTTGATTTTAAATTAGAAGC/ AATTTCCTTTACTTGTAGCAG | 377 | Outer | 66 | 78 | 79 | 90 |
| MhL044A_02730 | ATGAGTGAGCCAAACCACGCC/ TGCTCCGCTAGTTGGTCTGCC | 201 | Extracellular | 98 | - | - | 117 |
| MhL044A_02757 | ACTAATACCGTTACTGCTCAAC/ TTGATAAGATGAACCATTTGTAGA | 256 | Extracellular | - | - | 108 | 131 |
| MhL044A_02777 | GGTAGCGGAGGTTCGTCTT/ TTTTTTCTCGCTAACCATTATTGC | 356 | Unknown | 81 | 70 | 82 | 95 |

[a]for truncated genes the locus ID is followed by an underscore with the number of amino acids the product contains followed by a letter given serially for each division made.

[b]sequence tag added to the 5' end of forward primer: AAAAGCGCTGAAAACCTGATCGAAGGCCGT. Sequence tag added to the 5' end of reverse primer: TGGTGATGGTGGTGACCCCA

[c] dashes in columns indicates cases were relative immunoreactivity measured 0.

**Appendix B**

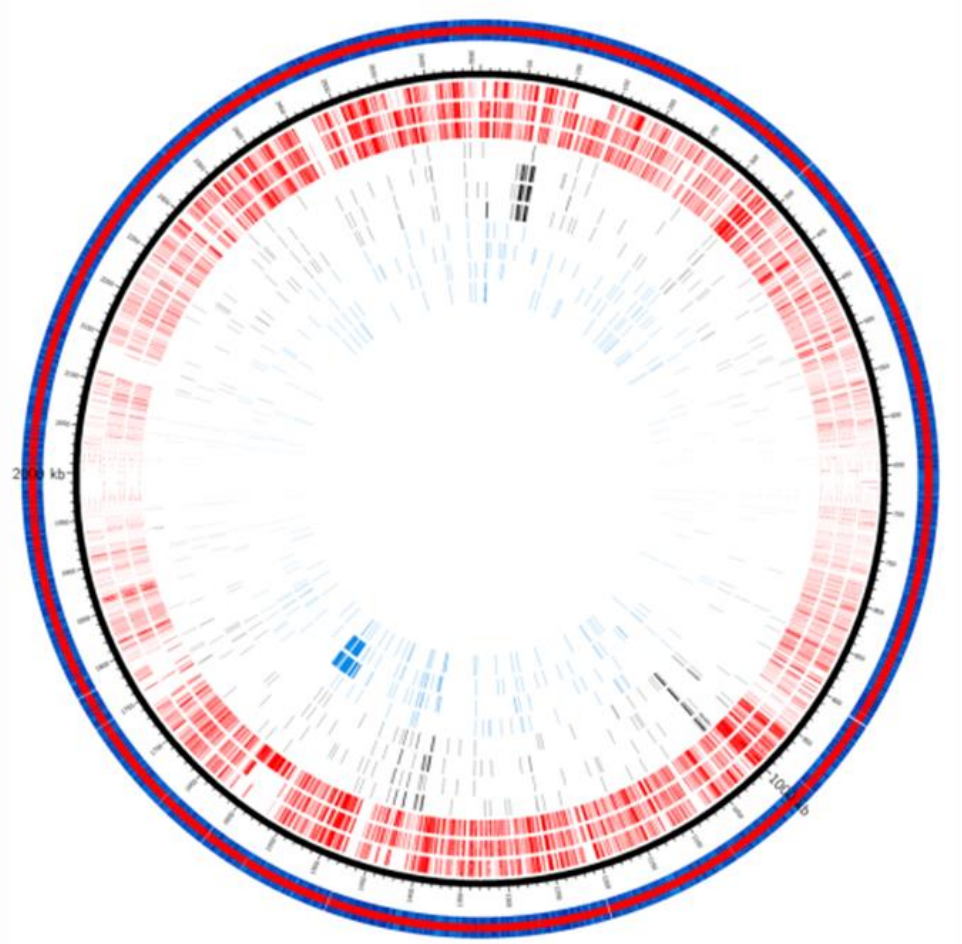

Figure B1 Circular SNP atlas of 11 *Mannheimia haemolytica* genomes mapped against *M. haemolytica* USDA-ARS-USMARC 183. The outer circle shows *M. haemolytica* USDA-ARS-USMARC 183 followed inwards by *M. haemolytica* 587A, *M. haemolytica* L033A, *M. haemolytica* T2, *M. haemolytica* L038A, *M. haemolytica* H23, *M. haemolytica* 3927A, *M. haemolytica* T14, *M. haemolytica* 157-4-1, *M. haemolytica* L044A, *M. haemolytica* L024A and *M. haemolytica* 535A.